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***The Impact of whey protein consumption and exercise on the  
composition and diversity of the gut microbiota: a high through-put  
DNA sequencing approach***

A thesis presented to the National University of Ireland for the degree of

Doctor of Philosophy

By

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## **Declaration**

I hereby certify that this material, which I now submit for assessment on the programme of study, leading to the award of PhD is entirely my own work and has not been submitted for another degree, either at University College Cork or elsewhere

Signed:

Student Number: 107581521

Date:

## **ABSTRACT**

Advances in culture independent technologies over the last decade have highlighted the pivotal role which the gut microbiota plays in maintaining human health. Conversely, perturbations to the composition or actions of the ‘normal/functioning’ microbiota have been frequently associated with the pathogenesis of several disease states. Therefore the selective modulation of enteric microbial communities represents a viable target for the development of novel treatments for such diseases. Notably, while bovine whey proteins and exercise have been shown to positively influence several physiological processes, such as energy balance, their effect on the composition or functionality of the gut microbiota remains largely unknown. In this thesis, a variety of *ex vivo*, murine and human models are used in conjunction with high-throughput DNA sequencing-based analysis to provide valuable and novel insights into the impact of both whey proteins and exercise on enteric microbial communities. Overall the results presented in this thesis highlight that the consumption both whey protein isolate (WPI), and individual component proteins of whey such as bovine serum albumin (BSA) and lactoferrin, reduce high fat diet associated body weight gain and are associated with beneficial alterations within the murine gut microbiota. Although the impact of exercise on enteric microbial communities remains less clear, it may be that longer term investigations are required for the true effect of exercise on the gut microbiota to be fully elucidated.

## Publications

Liam McAllan., **Peter Skuse.**, Paul D. Cotter., Paula O' Connor., John F. Cryan., R. Paul Ross., Gerald Fitzgerald., Helen M. Roche and Kanishka N. Nilaweera. (2014). Protein quality and the protein to carbohydrate ratio within a high fat diet influences energy balance and the gut microbiota in C57BL/6J mice. *PloS one 9, no. 2 (2014): e88904*

**Peter Skuse.**, Kanishka N. Nilaweera., Bettina McManus., Gerald F. Fitzgerald., Riitta Korpela., Paul D. Cotter. Voluntary exercise alters energy balance and the gut microbiota in a time dependent manner in C57BL/6 mice fed a low fat diet. *In review*

**Peter Skuse.**, Alice M. Moscovici., Kanishka Nilaweera., Gerald F. Fitzgerald., Uri Lesmes., Paul D. Cotter. Impact of the dairy whey proteins lactoferrin and serum albumin on the composition of the gut microbiota. *Manuscript ready for submission*

**Peter Skuse.**, Gerald Fitzgerald., Paul D Cotter. Bovine whey proteins: Agents to beneficially modulate the gut microbiota? *Manuscript ready for submission*

## List of Abbreviations

<sup>1</sup> HNMR	<sup>1</sup> H nuclear magnetic resonance
ACE	Angiotension-converting enxyme
BAMLET	Complex of $\alpha$ -lactalbumin and oleic acid
BCAAs	Branched chained amino acids
BCFAs	Branched chain fatty acids
BLAST	Basic local alignment search tool
BMI	Body mass index
BSA	Bovine serum albumin
CaCO <sub>3</sub>	Calcium carbonate
CCK	Cholecystokinin
CD	Crohn's disease
CD36	Cluster of differentiation 36
CPT1c	Carnitine palmitoyltransferase
CRC	Colorectal cancer
CREC	Clinical Ethics Commitee
DCA	Deoxycholic acid
DEXA	Dual energy X-ray absorptiometry
DGGE	Denaturant gradient gel electrophoresis
DIO	Diet induced obesity
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl peptidase 4
DW	Distilled water
EHEC	Enterohemorrhagic <i>E.coli</i>

EPEC	Enteropathogenic <i>E. coli</i>
ETBF	Enterotoxigenic <i>Bacteroides fragilis</i>
ETEC	Enterotoxigenic <i>E. coli</i>
Ex	Exercise group
EX+WPS	Exercise and whey protein supplementation group
<i>F:B</i>	Firmicutes : Bacteroidetes ratio
FABP	Fatty acid binding protein
FASN	Fatty acid synthase
FFAR2	Free fatty acid receptor 2
FFAR3	Free fatty acid receptor 3
FLASH	Fast length adjustment of short reads to improve genome assemblies
FOS	Fructo-oligosaccharide
GCCR	Glucocorticoid receptor
GF	Germ free
GHS-R	Growth hormone secretatogue
GI	Gastro-intestinal
GIP	Gastric inhibitory peptide
GIT	Gastro-intestinal tract
GLP-1	Glucagon-like peptide 1
GMP	Glycomacropeptide
GOS	Galacto-oligosaccharide
GRAS	Generally regarded as safe
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
H <sub>2</sub> S	Hydrogen sulphide

HCL	Hydrochloric acid
HFD	High fat diet
HO <sub>2</sub> SCN	Cyanosulphurous acid
HO <sub>3</sub> SCN	Cyanosulphuric acid
HOSCN	Hypothiocyanous acid
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
<i>IL10</i> <sup>-/-</sup>	Colitis-susceptible mice
Il-6	Interleukin-6
IL-8	Interleukin-8
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
Lf	Lactoferrin
Lfcin	Lactoferricin
LFD	Low fat diet
LP	Lactoperoxidase
LPS	Lipopolysaccharide
MGC	Metagenomic gene clusters
MRPs	Maillard reaction products
NaCl	Sodium chloride
NEFA	Non-esterified fatty acids
NOCs	N-Nitroso compounds
NPY	Neuropeptide Y
NSP	Non-starch polysaccharide
<i>Ob/Ob</i>	Genetically obese mice

ObR	Leptin receptor
OSCN <sup>-</sup>	Hypothiocyante
P/C	Protein to carbohydrate ratio
PCoA	Principal co-ordinate analysis
PCR	Polymerase chain reaction
PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PKS	Polyketide synthase
PMSF	Phenylmethanesulfonylfluoride
POMC	Pro-opiomelancortin
PPAR	Peroxisome proliferator activated receptor
QIIME	Quantitative Insights Into Microbial Ecology
RER	Respiratory exchange ratio
rhLf	Recominant human lactoferrin
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
RS	Resistant starch
SCFAs	Short chain fatty acids
SCN <sup>-</sup>	Thiocyanate anion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIBO	Small intestinal bacterial overgrowth
SIF	Simulated intestinal fluid
SIHUMI	Simplified human intestinal microbiota
SRB	Sulphate-reducing bacteria



T2D	Type-2-diabetes
TAG	Triacylcerol
TNBS	2,4,6 trinitrobenzenesulphonic acid
TNF $\alpha$	Tumour necrosis factor $\alpha$
UC	Ulcerative colitis
WHO	World Health Organisation
WPC	Whey protein concentrate
WPI	Whey protein isolate
WPS	Whey protein supplementation group
$\alpha$ -La	$\alpha$ -lactalbumin
$\beta$ -lac	$\beta$ -lactoglobulin

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## **Chapter 1**

### **Bovine whey proteins: Agents to beneficially modulate the gut microbiota?**

## 1.1 ABSTRACT

The significant advances in culture independent technologies over the past decade have allowed for a meteoric rise in our understanding of the structure, composition and function of the 100 trillion microbial cells which inhabit the human gut. The gut microbiota is now understood to play a pivotal role in maintaining human health by influencing several physiological processes, such as modulation of the host immune system, regulation of fat storage as well as conferring protection against opportunistic pathogens. Conversely, perturbations to the composition of the normal gut microbiota have been frequently associated in the pathogenesis of several gastrointestinal (GI) diseases such as obesity and inflammatory bowel disease. Therefore it is a reasonable supposition that selective modulation of the gut microbiota has the potential to deliver novel treatments for such disease states. Diet is a major driving force in determining gut microbiota composition with the balance of macronutrients within the diet differentially effecting gut microbiota composition. To date, efforts using diet to beneficially modulate gut microbial communities have mainly focused on non-digestible carbohydrates (prebiotics), whose fermentation results in the production of short chain fatty acids (SCFAs), while dietary protein sources have traditionally been associated with the formation of putrefactive metabolites which may be detrimental to gut health. With respect to protein, whey is the milk serum which remains after the casein curd has been removed during the cheese making process and contains ~20% of the total protein content in milk. Whey is comprised of several component proteins,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, serum albumin, lactoperoxidase, and lactoferrin, whose properties have been shown to beneficially influence several physiological processes such as energy balance and glucose metabolism. However, their effect on the composition of the gut microbiota

remains unclear. Here we review the role of the gut microbiota in GI diseases, the impact of diet on the gut microbiota and discuss properties of whey proteins which may represent novel agents by which enteric microbial populations may be modulated.

## 1.2 Introduction

Humans may be thought of as ‘superorganisms’ owing to the genetic potential encoded not only within our own genomes but also that encoded within the genomes of our resident microbial populations. It is estimated that over 1000 bacterial species are contained within the human microbiota, each of which possesses a genome encoding an average of ~5,000 genes (Consortium 2012). The human microbiota and associated metagenome therefore carries with it a genetic repertoire which far outweighs that of the human host (~23,000 genes) and is capable of carrying out metabolic functions not encoded by the human genome. The vast majority of these microbial inhabitants (1.5 kg or 100 trillion cells) reside within the gut (Lander et al. 2001, Eckburg et al. 2005, Consortium 2012). It has also been noted that while there are considerable inter-individual variations in the composition of the gut microbiota, there is a conserved set of metabolic functions shared between individuals known as the ‘core gut microbiome’ (Turnbaugh and Gordon 2009) suggesting that the overall functionality of the gut microbiota is of more importance to the host than its composition. The functions encoded within in the core gut microbiome are likely essential for optimum functionality of the gut. Studies have shown that the core gut microbiota confers several health benefits to the host which include protection against potential pathogens, regulation of fat storage and modulation of the host immune system (Sekiroy et al. 2010).

The composition of the gut microbiota goes through considerable changes throughout the life cycle of the host. Bacterial colonization of the GI tract occurs immediately following birth, where strict anaerobes such as *Bacteroides* spp., *Clostridium* and *Bifidobacterium* are amongst the initial colonizers upon depletion of the initial oxygen supply, although factors such as formula versus breastfeeding and



delivery method can have significant impacts on initial enteric bacterial populations (Palmer et al. 2007, Adlerberth and Wold 2009, Isolauri 2012). The formation of the ‘healthy’ adult gut microbiota begins from ~2 years of age and it is thought to remain relatively stable until undergoing a final shift upon entering the elderly phase of life. The stable adult gut microbiota is primarily comprised of bacterial species which are members of the Firmicutes and Bacteroidetes phyla, with the remaining species being members of the Actinobacteria, Verrucomicrobia, Proteobacteria, Fusobacteria and/or Cyanobacteria phyla (Qin et al. 2010). Upon entering old age, the gut microbiota undergoes a final shift which is associated with an increased abundance of *Bacteroides* spp. and distinct shifts within the *Clostridium* genus (Claesson et al. 2011). These age associated phylogenetic shifts are thought to result from changes in host physiology such as chronic low-grade inflammation and changes in dietary habits (Franceschi 2007).

Advances in culture independent, high throughput sequencing technologies and subsequent data analysis tools over the last decade have allowed for a dramatic rise in our understanding of the gut microbiota. Indeed, a PubMed search, using the term “gut microbiota”, for articles published between 2005 and 2015 returns 5240 related articles, whereas the same search yields just 144 related articles from the proceeding decade. On the basis of this new knowledge, the gut microbiota has been termed the body’s ‘forgotten’ organ (O'Hara and Shanahan 2006). In addition to contributing to health, perturbations of normal/healthy gut microbiota and functionality have also been shown to play a significant role in the pathogenesis of several GI diseases.

### **1.3 The role of the gut microbiota in gastrointestinal diseases**

There is an ever growing appreciation that perturbations in the composition of the normal gut microbiota contributes to the aetiology or severity of a substantial number of disease states ranging from Alzheimer's diseases to obesity and type 2 diabetes (T2D), reviewed comprehensively elsewhere (Clemente et al. 2012, de Vos and de Vos 2012, Carding et al. 2015). Indeed, such is the frequency that associations between disease states and the gut microbiota have been reported, this review will focus briefly on those between the gut microbiota and chronic GI diseases, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity, T2D and colorectal cancer (CRC), only. (Figure 1).

#### **1.3.1 Inflammatory bowel disease**

Ulcerative colitis (UC) and Crohn's disease (CD) are forms of inflammatory bowel disease (IBD) and are characterised by chronic relapsing inflammation of the GI tract (Podolsky 1991). While the aetiology of UC and CD are not fully understood, evidence suggests that both genetic and environmental factors play important roles in their development. Spehlmann *et al.* reported that while monozygotic twins were significantly more likely to be concordant for IBD than dizygotic twins, the genetic influence observed in the monozygotic twin cohort was relatively low (16% for UC and 35% for CD), highlighting the importance of environmental factors in disease pathogenesis (Spehlmann et al. 2008).

There is increasing evidence that a perturbations to the normal intestinal microbiota has a role in the pathogenesis of IBD, where IBD patients harbour an altered gut microbiota (Frank et al. 2007, Sokol and Seksik 2010). Patients with IBD typically display a reduced bacterial diversity (Elson and Cong 2012). Ott *et al.* demonstrated

that alterations to the gut microbiota may be more dramatic in UC than CD, where overall bacterial diversity was 50% lower in CD patients and 70% lower in patients with UC than in healthy controls. This reduced diversity was associated with a loss of commensal anaerobic bacteria such as *Bacteroides* species (Ott et al. 2004). Notably, metagenomic and metaproteomic studies have revealed that the microbiome of patients with IBD possess 25% fewer genes and are depleted in functional pathways and proteins when compared to healthy individuals (Qin et al. 2010, Erickson et al. 2012)

In terms of compositional alterations to the gut microbiota, patients with IBD have consistently been shown to have reduced diversity within the Firmicutes phylum, with fewer of its constituent bacterial species detected when compared to healthy individuals (Ott et al. 2004, Manichanh et al. 2006, Frank et al. 2007). Among the decreases within the Firmicutes phylum is a reduced abundance of the butyrate producing bacteria *Faecalibacterium prausnitzii* in both UC and CD patients (Sokol et al. 2008, Willing et al. 2009, Machiels et al. 2013). Indeed, Sokol *et al.* showed that oral administration of *F. prausnitzii* markedly reduced the severity of 2,4,6 trinitrobenzenesulphonic acid (TNBS) induced colitis and tended to correct the colitis induced microbial dysbiosis in mice (Sokol et al. 2008). It should, however, be noted that Hansen *et al.* have reported greater proportions of *F. prausnitzii* in a paediatric cohort of CD patients (Hansen et al. 2012) and so the role of *F. prausnitzii* in IBD among different age groups requires further attention.

IBD patients have been shown to harbour greater Proteobacteria concentrations when compared to healthy individuals and so it has been suggested that species belonging to this phylum, particularly pathogenic strains *Escherichia coli*, play an aggressor role in IBD (Gophna et al. 2006, Baumgart et al. 2007, Carvalho et al.

2009, Mukhopadhyaya et al. 2012). Indeed, increases in facultative anaerobes (such as the Proteobacteria) and decreases in obligate anaerobes (including many Firmicutes) reported in IBD patients have given rise to the so called “oxygen” hypothesis, which suggests that individuals with IBD have a disruption in the anaerobiosis of the GI tract resulting in the aforementioned alterations to the gut microbiota (Rigottier-Gois 2013).

Ultimately, it remains unclear whether the microbial dysbiosis observed in individuals with IBD is a cause of IBD or a consequence of the disturbed gut environment as a result of chronic inflammation. A recent study by Gevers *et al.* assessed the intestinal microbiota of a large cohort (n=447) of newly diagnosed paediatric CD patients. The authors reported that CD patients had increases in the abundance of *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae* and *Fusobacteriaceae* with concomitant decreases in abundances of *Erysipelotrichales*, *Bacteroidales* and *Clostridiales* when compared to a cohort (n=221) of healthy individuals implicating alterations to the gut microbiota in the development of CD. Notably, microbial differences between healthy controls and CD patients were only observed in mucosal samples (rather than fecal) suggesting alterations in bacteria which reside in the mucosal layer play an important role in the aetiology of the disease (Gevers et al. 2014).

### **1.3.2 Irritable bowel syndrome**

Irritable bowel syndrome (IBS) is a chronic function disorder of the GI system which is characterised by abdominal pain and altered bowel habits, wherein patients primarily experience diarrhoea, constipation or both (Canavan et al. 2014). While the underlying pathogenesis of this multifactorial syndrome is poorly understood

(Marshall et al. 2006), perturbations in the normal gut microbiota are thought to play a role in the low-grade inflammation associated with IBS (Öhman and Simrén 2013). Several studies have reported a microbial dysbiosis in patients with IBS when compared to healthy controls. Rajilic-Stojanovic *et al.* reported paediatric patients with IBS had increased populations of members of the Firmicutes phylum, namely *Ruminococcus*, *Clostridium* and *Dorea*, coupled with decreased populations of *Bifidobacterium* and *Faecalibacterium* spp (Rajilić–Stojanović et al. 2011). Similar results were also reported by Saulnier *et al.* who also reported an increase in Proteobacteria populations in a paediatric cohort with IBS (Saulnier et al. 2011).

Small intestinal bacterial overgrowth (SIBO) is defined as the presence of bacteria in excess of  $10^5$  CFU/ml of intestinal fluid (Posserud et al. 2007) and has been associated with the aetiology of IBS. SIBO results in the bacterial fermentation of dietary carbohydrates within the small intestine which increases gas production, contributing to the symptoms of IBS (Ghoshal et al. 2006). Furthermore, the production of SCFAs by bacteria associated with SIBO reduces intestinal motility by triggering the release of peptides such as peptide YY thus altering normal intestinal transit (Dumoulin et al. 1998).

A recent study which examined the fecal metabolite profiles using proton nuclear magnetic resonance concluded that samples from IBS patients and healthy controls could be distinguished based on their metabolite profile, with the intestinal environment of IBS patients being characterised by increased proteolysis, incomplete anaerobic fermentation and alterations in methane production. This study suggests that fecal metabolite and microbiota profiles are altered in IBS and may serve as biomarkers of human intestinal diseases (Shankar et al. 2015).

Both probiotic and antibiotic interventions have been observed to reduce the symptoms of IBS, further suggesting a role for bacteria in IBS pathogenesis (Collins 2014). The administration of the antibiotic rifaximin markedly reduced IBS symptoms bloating, abdominal pain and loose or watery stools compared with a placebo group in one such study (Pimentel et al. 2011). A probiotic study by Tsuchiya *et al.* demonstrated that IBS patients in receipt of a probiotic cocktail (SCM-III) containing *Lactobacillus helveticus* ( $1.3 \times 10^9/100\text{ml}$ ), *Lactobacillus acidophilus* ( $1.25 \times 10^9/100\text{ml}$ ) and *Bifidobacterium* ( $4.95 \times 10^9/100\text{ml}$ ) was either “effective” or “very effective” in more than 80% of patients. These patients displayed significant improvements in abdominal pain and bloating, which was concomitant with significantly increased abundances of intestinal *Lactobacillus*, *Eubacterium* and *Bifidobacterium* compared to a control cohort in receipt of a placebo (Tsuchiya et al. 2004). In a double-blind probiotic study, Kim *et al.* showed that administration of a the probiotic cocktail (Medilac DS) to IBS patients significantly reduced the frequency and severity of abdominal pain (Kim et al. 2006). These results indicate a bacterial role in IBS onset and thus therapeutic modulation of the gut microbiota as a viable target for IBS treatment.

### **1.3.3 Obesity**

According to the most recent WHO estimates, 1.9 billion adults (over 18) worldwide were overweight, of which nearly 600 million were obese (WHO 2015). Obesity is a multifactorial condition which primarily arises as a result of a long-term positive energy balance and is associated with a wide range of metabolic diseases such as type 2 diabetes (T2D), hypertension and cardiovascular disease (Eckel et al. 2005). While the dramatic increase in the incidence of obesity over the last 20-25 years to its current epidemic status is largely due to modern eating habits and an increasingly

sedentary lifestyle, increasing evidence has shown that the composition, diversity and function of the gut microbiota can play important roles in the aetiology of obesity. The relationships between the gut microbiota and obesity have been the focus of several comprehensive review articles (Flint 2011, Clarke et al. 2012, Cani 2013, Moran and Shanahan 2014).

Obesity is associated with an alteration in gut microbiota composition with a concurrent reduction in overall diversity (Turnbaugh et al. 2008, Turnbaugh et al. 2009). Several studies have reported that obese mice (Ley et al. 2005, Turnbaugh et al. 2006) and, to a lesser extent, humans (Ley et al. 2006) harbour increased proportions of the Firmicutes phylum with concurrent decreases within the Bacteroidetes phylum compared to their normal weight counterparts, leading to an increased Firmicutes : Bacteroidetes ( $F:B$ ) ratio being proposed as a potential biomarker of obesity. However, findings to the contrary have also been published (Murphy et al. 2010, Schwartz et al. 2010), including data from the Human Microbiome Project which indicated no association between the  $F:B$  ratio and obesity or body mass index (BMI) (Finucane et al. 2014) and, thus, the accuracy of the  $F:B$  ratio as a biomarker of obesity remains in question.

While the precise alterations in microbial composition that contribute to obesity remains the subject of debate, several important studies in gnotobiotic mice have highlighted the importance of the gut microbiota in the pathogenesis of obesity. Firstly, Backhed *et al.* demonstrated that the enteric microbiota is essential for the development of obesity in that the authors reported that germ free C57BL/6J mice were protected from diet induced obesity (DIO) whereas their conventionalised counterparts were not, despite no significant differences in energy (food) intake between the two groups (Bäckhed et al. 2007). Furthermore, Turnbaugh *et al.*

suggested that the obese-associated microbiota contributes to obesity through increased dietary harvest following observations that germ free mice conventionalised with a microbiota from genetically obese (*ob/ob*) mice had significantly higher amounts of body fat than mice conventionalised with a microbiota from lean animals (Turnbaugh et al. 2006). This theory was supported by a later study by Ridaura *et al.* who examined the effect of conventionalising germ free mice with the microbiota of twins discordant for obesity. The results of the study indicated that conventionalisation of germ free mice with a microbiota from obese twins (Ob) resulted in significant increases in body weight and adiposity when compared with mice conventionalised with a microbiota from lean twins (Ln). Moreover, co-housing of the Ob and Ln associated animals transformed the metabolic profile of the Ob associated animals to a Ln like state and prevented the development of increased body mass and adiposity (Ridaura et al. 2013).

More recent studies have identified specific bacterial members of the gut microbiota which may play important roles in obesity determination. Fei *et al.* demonstrated that the mono-association of germ free C57BL/6J mice with an endotoxin producing *Enterobacter* strain, namely *Enterobacter cloacae* B29, isolated from the gut of a morbidly obese human, induced fully developed obesity and insulin resistance whereas germ free mice consuming the same high fat diet did not display the same disease phenotype. *Enterobacter*-associated mice displayed aggravated inflammatory conditions and increased serum endotoxin load suggesting a causative role for the *Enterobacter* strain in the onset of obesity (Fei and Zhao 2013). Another study, by Woting *et al.*, revealed that *Clostridium ramosum* may also contribute to the aetiology of obesity. In this study, germ free mice were associated with a simplified human intestinal microbiota (SIHUMI) with or without *C. ramosum*



(SIHUMI+Cra or SIHUMI-Cra) or with just *C. ramosum* (Cra) and were provided with a high fat or low fat diet. After four weeks of high fat feeding, both SIHUMI+Cra and Cra mice displayed significantly higher body weight, body fat and higher food efficiency when compared to the SIHUMI-Cra animals. The authors of the study concluded that the presence of *C. ramosum* within the gut ecosystem intensifies diet induced obesity, possibly through the up regulation of small intestinal glucose and fat transporters (Woting et al. 2014). Conversely, other strains of commensal enteric bacteria such as *Enterococcus faecalis* FK-23 and *Lactobacillus gasseri* SBT2055 have been shown to exert anti-obesity and anti-adiposity effects in both rodent and human studies (Motonaga et al. 2009, Kadooka et al. 2010). The mucin degrading bacteria *Akkermansia muciniphila* has also received attention in this regard (Derrien et al. 2004). A study of note by Everard *et al.* demonstrated that, compared to their lean littermates, *ob/ob* mice had significantly lower abundances (3,300 fold) of *A. muciniphila*, which were normalised upon prebiotic (oligofructose) treatment, which in turn correlated with an improvement in the animal's metabolic profile. Furthermore, the same study showed that orally administered *A. muciniphila* reversed high fat associated metabolic disorders including fat mass gain and insulin resistance in these mice (Everard et al. 2013). Another study noted an increase in the abundance of *A. muciniphila* in a cohort of overweight/obese humans following a period of calorie restriction which coupled with an improvement in several clinical parameters such as insulin resistance (Dao et al. 2015). While data from these studies suggest a protective role for *A. muciniphila* in obesity, contrasting results were observed by Qin *et al.* who in a large metagenome wide association study of 345 Chinese individuals reported that *A. muciniphila* was enriched in patients with type 2 diabetes (Qin et al. 2012).

These results provide evidence that the gut microbiota plays an important role in the onset and severity of obesity and related conditions. These observations are particularly important in light of the fact that strategies to prevent/reduce the growing trend of worldwide obesity have been largely unsuccessful (Roberto et al. 2015), and suggest that the gut microbiota represents a viable alternate target in the treatment of obesity

#### **1.3.4 Type 2 diabetes**

T2D, which is primarily linked with obesity-related insulin resistance, has become a major health concern throughout the developed world (Chen et al. 2012). Obesity-related T2D is associated with the presence of low-grade inflammation in bodily sites involved in metabolism regulation such as the liver, adipose tissue and muscles (Pickup and Crook 1998). This low-grade inflammation results from the moderate excess in the production of cytokines such as interleukin (IL)-6 and tumour necrosis factor alpha (TNF- $\alpha$ ), resulting in alterations in insulin signalling and subsequent development of insulin resistance (Hotamisligil 2006, Shoelson et al. 2006).

Although obesity and associated inflammation are the primary cause of T2D, other factors such as host genetics (see review by Barroso (Barroso 2005)) and other environmental factors influence the condition. As with the aforementioned conditions, alterations to the gut microbiota in patients with T2D compared to healthy individuals have been reported in several studies (Larsen et al. 2010, Qin et al. 2012, Karlsson et al. 2013, Serino et al. 2013). Larsen *et al.* demonstrated that Bacteroidetes and Betaproteobacteria proportions were significantly enriched while proportions of the Firmicutes phylum, specifically within the *Clostridia* class were significantly decreased in adult males with T2D compared to their healthy

counterparts (Larsen et al. 2010). In a much larger study, Qin *et al.* concluded that the T2D associated microbiota was characterised by a moderate dysbiosis, an increase in the number of various opportunistic pathogenic bacterial species and a reduction in the levels of butyrate producing species (Qin et al. 2012). Similar results were reported by Karlsson *et al.* who, in a study examining the microbiome of type 2 diabetic European women, reported that four *Lactobacillus* species were enriched in the T2D diabetic cohort compared to the normal glucose tolerance control group, while conversely the abundance of five *Clostridium* species were decreased in the T2D cohort. Notably, the increases of *Lactobacillus* species and decreases in *Clostridium* species correlated positively and negatively respectively with fasting glucose, but did not correlate with BMI suggesting these alterations are related to the diabetic condition rather than the associated body weights of the subjects (Karlsson et al. 2013). Notably, when the same study analysed metagenomic gene clusters (MGCs), generated by shotgun sequencing of both their own cohort of European women and the Chinese cohort generated by Qin *et al.* (Qin et al. 2012), principal component analysis of MGC abundances of European and Chinese cohorts showed a clear separation which may reflect different genetics and/or different dietary habits. However, several striking similarities within the T2D-associated microbiota were also determined between the two groups, potentially revealing bacterial species that contribute to the development of T2D. More specifically, the metagenome of T2D patients in both European and Chinese cohorts were determined to have increased *Lactobacillus* and *Clostridium clostridioforme* MGCs and decreased *Rosburia\_272* MGCs. This increase in *Lactobacillus* is in agreement with previously mentioned study by Larsen *et al.*, who

also reported a positive correlation between the abundance of *Lactobacillus* and blood glucose levels in T2D patients (Larsen et al. 2010).

The reduced levels of *Roseburia* observed in both T2D cohorts may be critical as several of the bacterial species contained within the *Roseburia* genus (e.g. *Roseburia inulinivorans*) are amongst the most dominant butyrate producing members of the enteric microbiota (Aminov et al. 2006). Butyrate is the primary energy source for colonic enterocytes and has several beneficial impacts on host physiology which may be important in T2D, such as reducing intestinal inflammation and improvements in intestinal barrier function (Flint et al. 2007, Peng et al. 2007, Cani 2013). Indeed, gut microbiota transplantations from lean donors to individuals with metabolic syndrome have been shown to increase *Roseburia* populations, increase butyrate production and improve insulin resistance (Vrieze et al. 2012). Furthermore, the microbiome of both European and Chinese T2D cohorts showed enrichment in metabolic functions related to oxidative stress further suggesting a link between the pathogenesis of T2D and alterations to the normal gut microbial composition as high oxidative stress has previously been linked to a predisposition to diabetic complications (Kashyap and Farrugia 2011).

The present data shows that both composition and function of the gut microbiota is altered in T2D patients. While several bacterial species have been implicated in playing causative roles in T2D, environmental factors such as geographical location and dietary habits also influence the microbiota of T2D patients and thus larger human studies are required in order to develop novel diagnostic or therapeutic strategies.

### 1.3.5 Colorectal cancer

There has been a rapid increase in incidence of colorectal cancer (CRC) in recent years and CRC is now the leading cause of cancer-related mortality worldwide (Jemal et al. 2011). As with several other GI diseases, the gut microbiota is also thought to play a role in CRC (Rowland 2009, Chen et al. 2013). Wang *et al.* demonstrated a clear structural segregation between the gut microbiota of CRC patients versus healthy individuals. The study reported that, when compared to healthy volunteers, the microbiota of CRC patients were enriched in *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, *Streptococcus* and *Peptostreptococcus*. Variations within the *Bacteroides* genus were also observed in that *B. vulgatus* and *B. uniformis* were decreased and *B. fragilis* increased in CRC patients (Wang et al. 2012). Notably, the enterotoxin produced by certain *B. fragilis* strains (Enterotoxigenic *Bacteroides fragilis* (ETBF)) have been shown to contribute to CRC both by direct genotoxicity and by the stimulation of epithelial cell shedding and gamma-secretase-dependent E-cadherin cleavage (Wu et al. 2006, Wu et al. 2007). This cleavage in turn increases intestinal barrier permeability and alters  $\beta$ -catenin/Wnt signal transduction within intestinal epithelial cells, resulting in the proliferation and oncogenic transformation seen in CRC (Wu et al. 2003). Notably, as seen in T2D patient studies, Wang *et al.* also demonstrated that abundances of the *Roseburia* genus were significantly lower in CRC patients (Wang et al. 2012).

Several metagenomic studies comparing the microbiota composition of healthy individuals and CRC patients have identified the enrichment of representatives of the *Fusobacterium* genus in CRC patients (Castellarin et al. 2012, Kostic et al. 2012, Wang et al. 2012, Tahara et al. 2014). Furthermore, Kostic *et al.* showed that

*Fusobacterium nucleatum* can generate a pro-inflammatory microenvironment via the recruitment of tumour-infiltrating immune cells thus contributing to colorectal neoplasia progression (Kostic et al. 2013). *E. coli* have also been linked with CRC pathogenesis. Arthur *et al.* reported that the microbiota of colitis-susceptible (*IL10*<sup>-/-</sup>) mice harbour *E. coli* at levels that were ~100 fold higher than their wild-type counterparts. The same study also showed that azoxymethane treated *IL10*<sup>-/-</sup> mice mono-associated with *E. coli* NC101 developed invasive mucinous adenocarcinomas and that upon deletion of the polyketide synthase (*pks*) genotoxic island from the *E.coli* strain, tumour multiplicity and invasion were significantly decreased (Arthur et al. 2012).

Finally, as well as identifying specific members of the gut microbiota involved in the development of CRC, studies have shown that certain products of bacterial metabolism may also influence CRC. Hydrogen sulphide (H<sub>2</sub>S) is produced in the gut via the bacterial metabolism of diet-derived sulphate and other compounds such as sulphur amino acids by sulphate-reducing bacteria (SRB) (Magee et al. 2000). The production of H<sub>2</sub>S by the gut microbiota is of importance to CRC because H<sub>2</sub>S has been shown to be genotoxic to intestinal epithelial cells and, even at low concentrations (0.25-2mM), H<sub>2</sub>S can cause significant DNA damage to human cell lines (Attene-Ramos et al. 2007, Wallace 2007). Indeed CRC patients have previously been associated with an enrichment in SRB and higher stool sulphide levels suggesting that H<sub>2</sub>S production by members of the gut microbiota plays an important role in CRC (Kanazawa et al. 1996, Marchesi et al. 2011, Carbonero et al. 2012, Kostic et al. 2012).

It has also been proposed that the production of reactive oxygen intermediates (ROIs) such as hydrogen peroxide and extracellular superoxide, which can cause

DNA damage (Huycke et al. 2002), by members of the gut microbiota can also contribute to the genesis of CRC. More specifically, a study by Balamurugan *et al.* reported that CRC patients had lower levels of the butyrate producing bacterial species *Eubacterium rectale* and *F. prausnitzii* and concomitant relatively greater proportions of *Enterococcus faecalis* proportions which have been shown to produce genotoxic metabolic products such as superoxide (Huycke et al. 2002). The relationship between bacterial metabolites and CRC are reviewed in greater detail by Louis *et al.* (Louis et al. 2014).

The data reviewed highlights the importance of the gut microbiota in several GI diseases. Therefore gut microbiota modulation towards a more desirable state presents an attractive viable target in the development of novel therapeutic strategies.

#### **1.4. Diet and the gut microbiota**

It is now widely accepted that diet is a driving force in determining the composition of the gut microbiota and the relationship between diet and the gut microbiota has been the subject of several comprehensive review articles (Flint et al. 2012, Scott et al. 2013, Flint et al. 2015). The type and amount of the main dietary macronutrients, i.e. carbohydrates, proteins and fats, have been shown to have a profound effect on the composition of the gut microbiota and the bacterial metabolites which it produces.

##### **1.4.1 Carbohydrate**

Each day approximately 40 g of dietary carbohydrate escape digestion by host enzymes and enters the human colon (Cummings and Englyst 1991). The primary

forms of carbohydrate entering the colon include resistant starches (RS), non-starch polysaccharides (NSP) and oligosaccharides. Under the anaerobic conditions of the colon, SCFAs are the major end products of carbohydrate fermentation, of which butyrate, propionate and acetate are the major SCFA produced by the gut microbiota (Flint et al. 2012). SCFAs have been shown to influence several host physiological processes, firstly SCFAs are absorbed and used as an energy source by the host, with butyrate acting as the primary energy source for intestinal epithelial cells (Peng et al. 2007, Flint et al. 2015). SCFAs produced by members of the gut microbiota can influence host energy intake by acting as key signalling molecules for the G protein coupled receptors, free fatty acid receptor 2 (FFAR2) and free fatty acid receptor 3 (FFAR3). Activation of these receptors results in the production and release of gut hormones such as peptide YY and glucagon-like peptide-1 (GLP-1), which stimulate satiety (Brown et al. 2003, Miyauchi et al. 2010, Sleeth et al. 2010). SCFAs, and particularly butyrate, also exert anti-inflammatory effects on the host (Segain et al. 2000, Hamer et al. 2008). Indeed the gut microbiota of patients with inflammatory conditions such as UC have a marked reduction in butyrate producing species and oral administration of butyrate producing bacterial species such as *F. prausnitzii* has been shown to dramatically decrease the pathogenesis of inflammatory associated diseases (Sokol et al. 2008, Machiels et al. 2013). Several excellent reviews detailing the impact of SCFAs produced by the gut microbiota on the host have been published (Macfarlane and Macfarlane 2012, Scott et al. 2013, Tan et al. 2014).

#### *Resistant starches and non-starch polysaccharides*

Starch is a complex polysaccharide which is comprised of two glucose polymers, namely amylose and amylopectin. While the majority of dietary starch is digested within the small intestine, a variable fraction can evade host digestion and pass into



the colon. This dietary fibre is referred to as “resistant starch” (RS) (Macfarlane and Englyst 1986). RS is divided into four subgroups (RS1-RS4) based on its properties which allow it to escape digestion in the small intestine. These are plant cell wall polymers (type 1), granular structure (type 2), retrogradation (type 3) and chemical cross-linking (type 4) (Englyst et al. 1992). It is estimated that RS represents the largest diet-derived energy source for colonic bacteria (Nugent 2005).

In a randomised crossover intervention study, Abell *et al.* reported that a bacterial group closely related to *Ruminococcus bromi* was significantly increased when volunteers were switched from a normal diet to one high in RS (Abell et al. 2008). A further study examining the effect of a high RS diet, this time in adult female pigs, demonstrated that a diet high in RS resulted in significantly higher proportions of *F. prausnitzii* with concomitant reductions in potential pathogenic bacterial species such as *E. coli* and *Pseudomonas* spp. when compared to pigs in receipt of a diet high in digestible starch. Furthermore, when compared to pigs consuming the high digestible starch diet, both cecal and colonic SCFA concentrations were significantly higher in pigs consuming the diet high in RS (Haenen et al. 2013). In a dietary intervention study with obese men by Walker *et al.*, it was shown that changes to fecal bacterial populations occurred within three to four days after subjects had been switched to a diet supplemented with RS3. Among the changes to fecal bacterial populations, it was shown that the proportions of *R. bromii* and *E. rectale* were significantly enriched in response to RS3 (Walker et al. 2011). Another study, involving healthy individuals, found that increasing dietary RS2 led to increased proportions of *R. bromii* and *E. rectale* while increased RS4 consumption led to increased abundances of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* suggesting amylolytic bacteria species harboured within the gut

microbiota vary in their ability to utilise different types of RS (Martínez et al. 2010). This suggestion was further evidenced in a study by Ze *et al.* who investigated the activities of four dominant amylolytic human gut bacterial species in the presence of different RS substrates. The results indicated that when raw or boiled RS2 or RS3 was the substrate, *R. bromii* and *B. adolescentis* showed much greater amylolytic activity than either *Bacteroides thetaiotaomicron* and *E. rectale*. Moreover, the same study suggested that *R. bromii* may be a keystone bacterial species in the breakdown of RS in the colon, in that it was shown in co-culture to stimulate RS2 and RS3 utilisation by the other three bacterial species (Ze et al. 2012).

While the aforementioned study by Walker and colleagues (Walker et al. 2011) did not report a significant change in the gut microbiota in response to wheat bran (a non-starch polysaccharide (NSP)) supplementation, the amount of supplemented wheat bran was considerably less (~5-fold) than RS which might account for the inability of wheat bran to modulate microbial populations (Walker et al. 2011). Hedemann *et al.* observed a significant increase in fecal SCFA concentrations in rats fed various sources of non-digestible carbohydrates, such as RS and NSPs such as pectin and inulin, which positively correlated with a significant increase in the thickness of the mucous layer when compared to rats fed a fibre free diet (Hedemann et al. 2009). A recent study by Tap *et al.* reported that increased dietary fibre resulted in increased microbial richness associated with increased abundances of *Prevotella* and *Coprococcus* as well as increased expression of genes encoding carbohydrate-active enzymes on fibre (Tap et al. 2015). Notably, increases in *Prevotella* abundances have previously been associated with the long term consumption of a diet high in plant polysaccharides (discussed in greater detail below).

## *Prebiotics*

Prebiotics are defined as “selectively fermented ingredients that result in specific changes, in the composition and/or activity in the GI microbiota, thus conferring benefit(s) upon host health” (Gibson et al. 2010). The key properties which define a prebiotic are; 1) a food ingredient which escapes assimilation in the small intestine, 2) upon reaching the colon it can be fermented, 3) selective stimulation of bacterial species which confer a health benefit to the host (Gibson et al. 2010). The most commonly studied prebiotics are carbohydrates such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Macfarlane et al. 2008). To date, the spectrum of bacteria targeted with prebiotics has been relatively narrow, mainly focusing on stimulation of *Bifidobacterium* and *Lactobacillus* species, and this topic has been reviewed elsewhere (Manning and Gibson 2004, Gibson et al. 2010, Russell et al. 2011). Notably, however, prebiotic stimulation of bacterial such as *Bifidobacterium* may indirectly stimulate the growth of other beneficial bacterial species and/or increase production of beneficial microbial metabolites through bacterial cross-feeding. For example, prebiotic stimulation of *Bifidobacterium* populations leads to increased lactate production, which in turn can be converted to butyrate by other bacterial species such as *Eubacterium halii* (Duncan et al. 2004, Falony et al. 2006).

As noted above, recent studies have highlighted several other gut bacterial species, such as *R. bromii*, *R. intestinalis*, *E. rectale* and *F. prausnitzii*, which may also confer substantial health benefits upon the host and thus represent new viable targets for prebiotic stimulation. Of these bacterial targets, perhaps the most attractive is *F. prausnitzii*. As previously discussed, *F. prausnitzii* abundances are decreased in inflammatory GI diseases such as UC and CD and that the bacterium has been

shown to exert anti-inflammatory effects on the host (Sokol et al. 2008, Willing et al. 2009, Machiels et al. 2013). Ramirez-Farias reported that abundances of *F. prausnitzii* were significantly increased in subjects during a period of daily inulin (10g/d) supplementation compared to a control period without supplementation (Ramirez-Farias et al. 2009). Furthermore, *F. prausnitzii* have been shown to utilise pectin (Lopez-Siles et al. 2012), suggesting it is a suitable prebiotic substrate in the stimulation of *F. prausnitzii* growth within the large intestine.

More generally, several studies have reported a reduction in inflammatory markers after prebiotic supplementation suggesting that prebiotic intervention may reduce the severity of diseases such as obesity and T2D which are characterised by low grade inflammation. A study from Dehghan *et al.* showed that women with T2D who consumed an oligofructose-enriched inulin supplement (10g/d) over an eight week period exhibited a significant reduction in plasma lipopolysaccharide (LPS), fasting plasma glucose, interleukin-6 (IL-6) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) when compared to a control cohort consuming a maltodextran placebo (Dehghan et al. 2014). Similar results have also been reported in studies employing galacto-oligosaccharide (GOS) prebiotics. In a 12 week placebo (maltodextran)-controlled crossover study, overweight women (n=45) with increased risk factors associated with metabolic syndrome were provided a daily GOS supplement (5.5g/d). Prebiotic consumption increased fecal *Bifidobacterium* abundance with concomitant decreases in *Bacteroides* and opportunistic pathogens such as *C. histolyticum*. Prebiotic consumption also lead to a reduction in the inflammatory state (as determined by fecal calprotectin and plasma CRP), and significantly decreasing total cholesterol and plasma tri-glyceride concentrations (Vulevic et al. 2013). Furthermore, other

studies have reported that consumption of prebiotics improved symptoms in UC and CD patients (Lindsay et al. 2006, Casellas et al. 2007, Zachos et al. 2007).

#### **1.4.2 Protein**

Each day approximately 15g of protein reaches the human colon. However, its impact on the colonic bacteria is currently poorly understood (Cummings 1997). Dietary protein is crucial for the supply of amino acids and also serves as the major nitrogen source for the growth of saccharolytic bacteria within the gut (Cummings and Macfarlane 1991). Compared to carbohydrate fermentation, bacterial metabolism of protein results in a more diverse range of metabolites, some of which are putrefactive and thus a predominantly saccharolytic gut environment is generally regarded as preferable (Conlon and Bird 2014, Rastall and Gibson 2015).

The primary pathway of protein fermentation occurring in the human colon is amino acid deamination, resulting in the formation of SCFAs and ammonia. Bacteria known to carry out these reactions include species within the *Clostridium*, *Bacteroides*, *Enterobacterium* and *Lactobacillus* genera (Cummings and Macfarlane 1991). It is thus unsurprising that ammonia, which has a normal fecal concentration of ~15mM, has been shown to increase in response to high protein intake (Hughes et al. 2000). Increased ammonia production in the gut may have implications for diseases such as CRC as ammonia has been shown in rat models to be a potent carcinogenic agent even at low concentrations (Windey et al. 2012). In addition to ammonia, protein fermentation by members of the gut microbiota also results in the formation of other detrimental metabolites, e.g. phenylacetic acid, phenols, indoles and p-cresol, implicated in the pathogenesis of diseases such as UC, CD and CRC

(Cummings and Macfarlane 1991, Jantchou et al. 2010). This topic is reviewed in greater detail by Windley *et al.* (Windey et al. 2012).

Other nitrogenous compounds produced through bacterial fermentation of dietary protein, particularly N-nitroso compounds (NOCs), may also be detrimental to gut health. A study by Russell *et al.* showed that during a period of low carbohydrate/high protein consumption, volunteers displayed an increase in NOC and branched chain fatty acids (BCFAs) concentrations with a concurrent decrease in beneficial microbial products such as butyrate (Russell et al. 2011). NOCs can be formed endogenously by the nitrosation of amines derived from the microbial fermentation of dietary protein in the colon and gut bacteria such as several members of the Proteobacteria phylum, which encode nitrate reductases, are thought to contribute to these nitrosation reactions. NOCs are potent carcinogenic agents which promote tumour formation *via* DNA alkylation leading to mutagenesis (Gill and Rowland 2002).

To date only a small number of studies have examined the effect of dietary proteins on the enteric microbiota and tend to focus on altered bacterial metabolites rather than specific taxonomic changes. However, it has been shown that individuals on a high protein/low carbohydrate diet for 4 weeks displayed altered gut microbiota and fatty acid profiles, which included a reduction in *Roseburia* and *E. rectale* and in colonic butyrate levels (Russell et al. 2011). The limited data available also suggests that the bacterial metabolites formed by proteolytic fermentation are highly dependent on the type of protein ingested. For example, in a series of studies by Tolden *et al.* rats consuming high protein diets, where the protein source was casein (25%), white meat (25%), red meat (35%) or soya (25%), displayed significantly higher colonocyte genetic damage than was the case for animals consuming a more

typical protein diet (15% casein). Notably, this detrimental effect was absent when whey protein (25%) was the protein source in a high protein diet (Toden et al. 2007, Toden et al. 2007, Toden et al. 2007). These studies also further highlighted the importance of the composition of the protein source in that, for example, red meat was shown to induce greater genetic damage than white meat, which was attributed to red meat containing higher levels of heme, which has previously been shown to stimulate the production of n-nitroso compounds within the human gut (Kuhnle et al. 2007). Moreover, animal protein was shown to cause DNA damage, which was associated with thinning of the colonic mucous barrier layer, to a significantly higher degree than plant or dairy protein (Toden et al. 2007). Substitution of casein as the dietary protein source with a less digestible protein source was shown to significantly increase intestinal BCFA concentrations and excretion of urinary p-cresol which was associated with increased tumorigenesis (Le Leu and Young 2007).

Finally, murine and human intervention studies have shown that administration of prebiotics and/or probiotics can exert a shift in gut microbial metabolism from proteolytic to saccharolytic fermentation in that urinary p-cresol and urinary nitrogen concentrations were observed to decrease in rats upon administration of RS (Heijnen and Beynen 1997, Toden et al. 2005). In a randomised crossover study Birkett *et al.* also demonstrated that high RS consumption significantly lowered fecal concentrations of ammonia and phenols (associated with protein fermentation) (Birkett et al. 1996). Ndagijimana and colleagues used  $^1\text{H}$  nuclear magnetic resonance ( $^1\text{H}$ NMR) to show that the consumption of a synbiotic (combined prebiotic and probiotic) food (0.5g FOS,  $10^9$  CFR *Bifidobacterium longum* and  $10^9$  CFU *Lactobacillus acidophilus* x2/day) for 30 days resulted in a metabolite profile

shift more indicative of saccharolytic fermentation rather than proteolytic fermentation (Ndagijimana et al. 2009). These results are in agreement with other synbiotic intervention studies (Oberreuther-Moschner et al. 2004, De Preter et al. 2011).

The current data indicates that utilisation of dietary protein by the gut microbiota is associated with increased production of metabolic end-products which are detrimental to gut health. However, the effect by which the amount and type dietary protein entering the colon exerts on specific taxonomies within the gut microbiota is still poorly understood and requires further investigation.

### **1.4.3 Fat**

The majority of dietary fat is adsorbed in the human small intestine. However, using  $^{13}\text{C}$  labelled fat, Gabert and colleagues showed that ~7% of dietary fat escapes the digestive process and is excreted in faeces. Thus, a substantial amount of fat reaches the colon and may influence the gut microbiota (Gabert et al. 2011). Several high fat diet associated rodent studies have suggested that alterations to the composition of the gut microbiota results from dietary fat rather than the obese phenotype. Zhang *et al.* reported that mice fed a high fat diet for 12 weeks to induce obesity harboured a gut microbiota considerably different to that of control mice consuming a normal chow diet. However, when the obese mice were switched to a normal chow diet for a further 10 weeks, the composition of their gut microbiotas became indistinguishable from those within the control cohort despite having significantly higher body weights even after the 10 week normal chow period. Thus the strong response of the gut microbiota to the high fat diet was reversible and was independent of the obese phenotype (Zhang et al. 2012). These results are in accordance with another study in



which RELM $\beta$  knockout mice in receipt of a high fat diet did not develop obesity but did display significant differences in gut microbial populations in comparison to their normal chow fed counterparts (Hildebrandt et al. 2009). When examining the results of these comparison studies, it is important to account for the variability in the macronutrient ratios of the diets used. For example, high fat diets are normally lower in carbohydrates and so the possibility exists that reported alterations to the gut microbiota of animals consuming a high fat diet in comparison to those on control diets may stem from differing amount of fermentable carbohydrates within the diet (Neyrinck et al. 2011, Scott et al. 2013).

High fat diets have also been shown to increase the production and release of bile acids microbiota such as deoxycholic acids (DCA) from the gall bladder and increase the concentrations of secondary bile acids produced by 7  $\alpha$ -dehydroxylation of primary bile acids by members of the gut microbiota (Rafter et al. 1987). Notably, the supplementation of a murine diet with DCA was shown to alter the composition of the gut microbiota, where an increased *F:B* ratio and an outgrowth of several bacteria within the *Erysipelotrichi* and *Clostridia* classes were reported (Islam et al. 2011). Furthermore, increased production of secondary bile acids by the gut microbiota has been implicated in pathogenesis of GI diseases such as CRC (Ou et al. 2012).

Finally, alterations to the gut microbiota in response to high fat/low carbohydrate diets are thought to contribute to low grade inflammation, characterised by increased levels of circulating LPS and other plasma inflammatory markers which have been shown to be mitigated after treatment with prebiotics such as FOS or arabinoxylans (Cani et al. 2007, Moreira et al. 2012). Supplementation of a high fat diet with FOS

was shown to restore *Bifidobacterium* spp. populations, reduce the expression of pro-inflammatory cytokines and improve glucose tolerance (Cani et al. 2007). Neyrinck *et al.* showed an improvement in circulating levels of IL-6 and MCP-1 following supplementation of a high fat murine diet with the prebiotic arabinoxylan, which was accompanied by a restoration of *Roseburia* populations and an enrichment of *Bifidobacterium* (Neyrinck et al. 2011). The increase in levels of circulating inflammatory markers resulting from high fat feeding has been associated with an increase in gut barrier permeability and, thus, increasing butyrate producing bacteria such as those within the *Roseburia* genus may ameliorate these symptoms, given that butyrate improves intestinal barrier function (Peng et al. 2007, Peng et al. 2009). Indeed, Cani *et al.* showed that supplementation of a high fat diet with the prebiotics FOS and arabinoxylan resulted in a selective increases in *Lactobaccillus* and *Bifidiobacterium* populations as well as the butyrate producing *C. coccides-E. rectale* cluster, which was accompanied with an improvement in intestinal barrier function mediated through a microbially driven increased production of GLP-2 (Cani et al. 2009).

#### **1.4.4 Impact of long term dietary patterns on the gut microbiota**

Metagenomic studies comparing the gut microbiota of cohorts from different geographical locations where the habitual long term dietary patterns are significantly different have highlighted the importance of the diet in determining the microbiota structure. In a study of 98 US adults, Wu *et al.* linked long term dietary habits with bacterial enterotypes, where the microbiota of individuals consuming higher amount of dietary fibre (rich in fruits and vegetables) were associated with the *Prevotella* enterotype whereas the microbiota of individuals consuming with a more ‘western’

diet higher in protein and fat were associated with the *Bacteroides* enterotype (Wu et al. 2011).

These results are in agreement with those reported in a comparison study of children from a rural African village in Burkina Faso and European children. The diet of the children from Burkina Faso, akin to that of early human settlements, was high in plant polysaccharides, starch and fibre and low in animal protein and fat. Comparatively, European children consumed a typical 'western' diet, high in animal protein, sugar, starch and low in fibre. Analysis of the fecal microbiota revealed distinct differences in the structure of the European and African childrens' gut microbiota. The gut microbiota of Burkina Faso children contained significantly lower proportions of Firmicutes and were significantly enriched in the *Prevotella* and *Xylanibacter* genera, which encode several xylan and cellulose hydrolysis genes, when compared to their European counterparts. This data suggests that the human gut microbiota co-evolved with a polysaccharide-rich diet allowing for increased energy extraction from the diet but also indicating that the microbiota may confer protection to the host against inflammatory and non-infectious colonic disease through increased SCFA production and, given the significantly higher levels of *Enterobacteraceae* harboured by European children, may also confer protection to the host against opportunistic gut pathogens (De Filippo et al. 2010).

In another study, the gut microbiota of city dwelling African Americans, which have a markedly increased risk of CRC, were compared by Ou and colleagues with native Africans with a very low incidence of CRC. 16S rRNA gene sequence data revealed distinct differences in microbial composition between the two cohorts, with a predominance of the *Prevotella* enterotype in the native African populations and of *Bacteroides* in the African American cohort. Furthermore, the native African

population were noted to have significantly higher levels of SCFAs while other bacterial metabolites associated with CRC, such as secondary bile acids, were significantly higher in the African American cohort (Ou et al. 2013).

Claesson *et al.* reported that differences in dietary patterns were responsible for the variations in gut microbial populations observed between community dwelling elderly individuals and those residing in long term care facilities. The study noted that community dwelling individuals which consumed a healthier and more diverse diet (low to moderate fat and high fibre), harboured a more diverse gut microbiota than individuals living in residential care. As well as a reduction in overall biodiversity, taxonomic differences were also detected between long stay care and community dwelling individuals, where the microbiota of community dwelling individuals were associated with greater abundances of *Prevotella*, *Coprococcus* and *Roseburia* and lower abundances of *Parabacteroides*, *Eubacterium*, *Anaerotractus*, *Lactonifactor* and *Coprobaecillus* than those individuals residing in long term care facilities (Claesson et al. 2011). The authors of the study also linked the observed alterations in gut microbial composition to length of stay, i.e. the longer the individual stayed in the resident care facility, and thus consumed a less diverse diet, the more dissimilar their microbiota became to that of the community dwelling individuals (Claesson et al. 2011).

Finally, a number of studies have highlighted differences in the fecal microbiota of individuals consuming vegetarian or omnivorous diets (Liszt et al. 2009, Kabeerdoss et al. 2012, Matijašić et al. 2014). Results from these studies indicated that the increased carbohydrate and fibre intake associated with vegetarianism results in increased SCFA production by enteric microbes, which lowers intestinal pH and may inhibit the growth of GI pathogens such as *E. coli* and other *Enterobacteriaceae*

spp. (Zimmer et al. 2012). However when compared to vegetarians, the gut microbiota of omnivorous individuals were shown to harbour an increased abundance of the *Clostridium* XIVa class which contains several important butyrate producing bacterial strains such as *R. inulinivorans*, *E. rectale* and *E. hallii* (Liszt et al. 2009, Louis and Flint 2009, Kabeerdoss et al. 2012).

Taken together, these studies highlight that diet plays a key role in enteric microbial structure and function. Pioneering work in the development of pre, pro and synbiotic treatments have shown that dietary interventions can beneficially modulate the gut microbiota to confer a health benefit on the host *via* such mechanisms as stimulating beneficial microbial populations and increasing SCFA production, which has in turn paved the way for the development of non-pharmaceutical, novel treatments for diseases such as T2D and IBS (Ringel et al. 2012, Rastall and Gibson 2015). Other studies have highlighted other bacterial species, such as *F. prausnitzii*, whose stimulation or probiotic administration may confer further health benefits to the host (Conlon and Bird 2014). However, current knowledge regarding the impact of macronutrient sources, particularly different protein sources, on specific members of the gut microbiota remains extremely limited. Certainly, when compared to carbohydrate utilisation by the gut microbiota, proteolytic fermentation results in the formation of several potentially putrefactive metabolites. However, the type of protein consumed appears to play a pivotal role in determining fermentation end products, for example whey protein was observed to result in less colonocyte DNA damage when compared to other protein sources (Toden et al. 2007) and therefore dietary proteins may represent an “untapped” resource in stimulating beneficial bacterial species or inhibiting potential pathogens.

Advances in technology and application of transcriptomic and metabolomic approaches in combination with existing metagenomic data promises to further advance our understanding of the complex relationship between diet and the gut microbiota and allow for the development of novel methods of gut microbiota modulation.

### **1.5 Bovine milk proteins as novel beneficial modulators of the gut microbiota?**

The consumption of dairy whey proteins, and their associated bioactive peptides, has been associated with a wide range of health benefits, however their impact on enteric microbial populations remains largely understudied. In this section we review the properties of bovine whey proteins, their influence on physiological processes and discuss the current knowledge on their impact on the gut microbiota.

#### **1.5.1 Bovine whey proteins**

Whey is the milk serum which remains after casein curd, formed by the action of chymosin [rennet], has been separated from the milk during the cheese making process (Figure 2). Whey is comprised of proteins, vitamins, lactose (~75% of total whey solids), minerals and small amounts of fats (Zadow 1994). Caseins account for 80% (W/W) of the total protein content in milk with whey containing the remaining 20%. Unlike proteins from the casein fraction of milk proteins, whey proteins do not coagulate in the acid conditions of the stomach and thus reach the jejunum quickly, are rapidly digested, can influence several metabolic functions such as lipid and glucose metabolism and can act as therapeutic agents in the treatment of diseases such as hypertension and cardiovascular disease (Hulmi et al. 2010). Traditionally thought of as a waste by-product of the cheese manufacturing process, whey is now regarded as a valuable co-product with several commercial applications.

## **1.6 Whey component proteins**

Whey is comprised of several component proteins including  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, bovine serum albumin, lactoperoxidase, lactoferrin (each discussed in greater detail below) as well as other minor proteins such as immunoglobulins. Whey is a high quality protein source and contains all essential amino acids, including high concentrations of branched chained amino acids (BCAAs) such as leucine, isoleucine and valine, which are important factors in biological processes such as muscle repair (Millward et al. 2008, Hulmi et al. 2010).

### **1.6.1 $\beta$ -lactoglobulin**

$\beta$ -lactoglobulin ( $\beta$ -lac) is the major protein in whey, accounting for about half of the total whey protein content and ~12% of total bovine milk proteins.  $\beta$ -lac is a globular protein which consists of 162 amino acid residues in its primary structure and has a molecular weight of 18.4 kDa (Eigel et al. 1984). While  $\beta$ -lac is the major protein of bovine whey, it is essentially absent from the milk of both humans and rodents. Indeed,  $\beta$ -lactoglobulin is the primary allergen in cow's milk and its removal from whey has been suggested for the production of hypoallergenic food products such as milk formula (Chiancone and Gattoni 1991). As well as providing a rich source of both essential and branched chain amino acids (Harper 2004),  $\beta$ -lac was also shown (in preruminant calves) to play an important role in the intestinal uptake of small hydrophobic ligands such as retinol, triglycerides and long chain fatty acids (Kushibiki et al. 2001). While not known for its antimicrobial activity,

proteolytic digestion of  $\beta$ -lac by trypsin results in the formation of four negatively charged peptides that possess bactericidal effects against Gram positive bacteria (Pellegrini et al. 1999). Notably,  $\beta$ -lac has also been proposed as a vehicle for the delivery of prebiotic compounds, viable probiotic bacteria and pH-sensitive drugs to the colon owing to  $\beta$ -lac's high resistance to gastric digestion, GRAS (generally regarded as safe) status and relative in-expense (Livney 2010, Mehraban et al. 2013, Kent and Doherty 2014).

### **1.6.2 $\alpha$ -lactalbumin**

Bovine  $\alpha$ -lactalbumin ( $\alpha$ -La), which consists of 123 amino acid residues, is the second most abundant protein found in whey and accounts for ~20% of the total protein content of bovine whey.  $\alpha$ -La is synthesised in the mammary gland where it acts as a coenzyme in the biosynthesis of lactose, an important energy source for the new-born (De Wit 1998).  $\alpha$ -La contributes to a reduced risk of incidence of some cancer types through several mechanisms such as the anti-proliferative effects demonstrated in assays with colon adenocarcinoma cell lines (Sternhagen and Allen 2001). Indeed, BAMLET, which is a complex of bovine  $\alpha$ -La and oleic acid, was shown by Rammer *et al.* to have the ability to kill tumour cells which are highly resistant to apoptosis (Rammer et al. 2010).

Due to its particularly high tryptophan content, diets enriched in  $\alpha$ -La also have beneficial effects with respect to mood and cognition. Tryptophan is a precursor to 5'-hydroxytryptamine, also known as serotonin, a neurotransmitter involved in several important neuro-processes such as sleep regulation and cognitive processes (Jouvet 1999). Indeed, a decreased serotonin level is associated with mood disorders such as anxiety and depression (Maes and Meltzer 1995). A human study by Markus



*et al.* demonstrated that evening intake of  $\alpha$ -La resulted in improved brain-sustained attention processes, which were associated with increased plasma tryptophan availability (Markus et al. 2005). In another study, rats consuming a  $\alpha$ -La enriched diet showed enhanced serotonin release resulting in the induction of anxiolytic and rewarding effects within the brain suggesting that a  $\alpha$ -La enriched diet may have a beneficial effect on mood (Orosco et al. 2004). When examined against several Gram positive and Gram negative bacteria, native bovine  $\alpha$ -La did not exhibit any bactericidal activity (Pellegrini et al. 1999), however Pihlanto-Leppälä *et al.* demonstrated that  $\alpha$ -La reduced the metabolic activity of *E. coli* JM103 by 79% when pre-hydrolysed with pepsin or trypsin (Pihlanto-Leppälä et al. 1999). Furthermore, Brück *et al.* showed that a combination of  $\alpha$ -La and glycomacropeptide inhibited the association between CaCo-2 cells and the GI pathogens enteropathogenic *E. coli* (EPEC), *Salmonella typhimurium* and *Shigella flexneri*, thus suggesting milk supplementation with both  $\alpha$ -La and GMP might be effective in the prevention of some GI infections (Brück et al. 2006).

### **1.6.3 Bovine serum albumin**

Unlike other whey proteins, bovine serum albumin (BSA) is not synthesised in the mammary gland but instead enters milk following its leakage from the blood stream and thus may be considered a whey associated protein. BSA, which contains 583 amino acid residues, is involved in the maintenance of colloid osmotic pressure and also acts as a carrier protein for free fatty acids, catecholamines and other hormones (Danon and Sapira 1972, Hankins 2006). While a good source of essential amino acids, there is currently little knowledge on the therapeutic potential of BSA. One notable property associated with the protein was its ability to inhibit tumour growth

in a MCF-7 human breast cancer cell line (Laursen et al. 1989). BSA may also beneficially influence fat mass accumulation as a recent study by McManus *et al.* (2015) reported C57BL/6 mice which had consumed a high fat diet where BSA was the dominant dietary protein source for 13 weeks had significantly reduced subcutaneous fat mass, plasma leptin and plasma corticosterone relative to animals which consumed a high fat diet where casein was the dominant dietary protein source.

#### **1.6.4 Lactoferrin**

Lactoferrin (Lf) is a single chained iron-binding member of the transferrin protein family and accounts for ~1% of the total proteins in bovine whey. Lf is produced by mucosal epithelial cells in several mammalian species and is found in mucosal secretions such as tears, saliva and GI fluids (Van der Strate et al. 2001, Öztafl and Özgünefl 2005). Of all the proteins contained within bovine whey, Lf is the most widely studied. Lf is a multifunctional protein whose properties include anti-bacterial activity and stimulation of beneficial intestinal bacteria (discussed presently), as well as other functional properties such as anti-viral, immunomodulatory, anti-cancer and anti-inflammatory activities (reviewed elsewhere (Van der Strate et al. 2001, Madureira et al. 2007, Garcia-Montoya et al. 2012)). Due to the wide range of beneficial properties associated with this whey protein, Lf may now be thought of as a nutraceutical protein and strategies have been developed to facilitate its use in nutritional and pharmaceutical applications.

While multiple functional properties of Lf have been investigated, its antimicrobial activity is its most widely studied function and both *in vitro* and *in vivo* studies have reported Lf activity against both Gram positive and Gram negative bacteria. In terms

of its antibacterial activity, Lf exerts both bacteriostatic and bactericidal activity. Lf comprises two homologous lobes, each of which can bind one ferric ion ( $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$ ) (Baker et al. 2003, Adlerova et al. 2008). Lf therefore exerts bacteriostatic activity through the sequestration of iron away from bacterial pathogens, thus limiting the use of this vital nutrient and down-regulating expression of virulence factors (Arnold and Cole 1977, Arnold et al. 1980, Reyes et al. 2005). Studies have shown that Lf also invokes bactericidal effects on pathogenic bacteria through direct interactions with bacterial surface proteins. Ellison *et al.* were the first to report that Lf damaged the outer membrane of Gram negative bacteria through interactions with lipopolysaccharide (LPS) (Ellison et al. 1988). More specifically, the damage to the LPS layer was shown to result from the binding of Lf to lipid A of LPS, causing structural changes and loss of membrane potential and integrity. The binding of Lf to LPS also reduces LPS-induced toxicity (Miyazawa et al. 1991) and can inhibit the adherence of several enteric pathogenic bacteria including *Salmonella*, enterotoxigenic *E. coli* (ETEC), EPEC, and enterohemorrhagic *E. coli* (EHEC) to intestinal cells thereby negating their ability to establish infection (Ellison et al. 1988, Appelmelk et al. 1994, Kawasaki et al. 2000, Ochoa et al. 2003, Ochoa and Cleary 2009). Interestingly, the binding of Lf to LPS and other bacterial surface proteins also potentiates the action of other antibacterial enzymes, such as lysozyme, or certain antibiotics, such as novobiocin (Ellison et al. 1988, Sanchez and Watts 1999).

The proteolytic enzymes found in the GI tract and sites of infection catalyse the release of several functional peptides from native Lf which confer anti-bacterial activity. Of these anti-bacterial peptides, which include lactoferrampin and LF1-11, the most widely studied is lactoferricin (Lfcin). Lfcin, which represents amino acid

residues 17-41 of native Lf, is a 25 amino acid residue multifunctional peptide resulting from the pepsin cleavage of native Lf. Lfcin contains two cysteine residues which are linked by a disulphate bridge, creating a looped region which contains many hydrophobic and positively charged residues (Bellamy et al. 1992). Bellamy *et al.* reported that bovine Lfcin exerts antibacterial activity against a wide range of both Gram positive and Gram negative bacteria and that its bactericidal effects were more potent than that of native Lf (Bellamy et al. 1992). Indeed, in a comparison study of human, bovine, murine and caprine Lfcin by Vorland *et al.*, bovine Lfcin was shown to be the most potent (Vorland et al. 1998). The bactericidal activity of Lfcin is derived from its ability to bind to the LPS layer of Gram negative bacteria (Yamauchi et al. 1993) and teichoic acid found within the cell wall of Gram positive bacteria (Vorland et al. 1999), resulting in membrane disruption, inhibition of macromolecular biosynthesis and, ultimately, cell death (Ulvatne et al. 2004). Kuwata *et al.* reported that Lfcin was produced in the human stomach following ingestion of bovine lactoferrin in physiologically functional quantities suggesting that Lfcin may play an important defence role within the GIT and that functional foods enriched with bovine lactoferrin may reduce enteric bacterial infection through increased Lfcin production (Kuwata et al. 1998). Notably, while Lfcin possesses potent anti-bacterial activity against a wide range of bacteria, certain bacterial species, some of which have been associated with improving gut health such as *Bifidobacterium bifidum* strains, are highly resistant to Lfcin (Bellamy et al. 1992, Khailova et al. 2009). Indeed, with respect to *Bifidobacterium*, Lf may also be thought of as a growth promoter of several strains of *Bifidobacterium*. This is particularly notable given the probiotic properties associated with many strains from this genus, as noted above and reviewed elsewhere (Russell et al. 2011).

Several *in vitro* studies have shown that bovine Lf (BLf) promotes the growth of several *Bifidobacterium spp.*. Saito *et al.* reported that both apo-BLf (iron free) and holo-BLf (iron saturated) exhibited bifidogenic activity with respect to eight strains of *Bifidobacterium*, with a considerable bifidogenic effect being reported for *B. breve* ATCC 15700 and *B. bifidum* ATCC 15696 (Saito et al. 1996). Furthermore, Rahman *et al.* reported that both apo-BLf and holo-BLf promote the growth of fourteen strains of *B. infantis*, *B. breve*, *B. bifidum* and *B. longum* with the bifidogenic effect of holo-BLf noted to be slightly more effective than that of apo-BLf (Rahman et al. 2010). Kim *et al.* suggested that the bifidogenic activity of Lf is dependant on the ability of the bacterial strains to bind Lf. More specifically, the authors reported that Lf increased growth of three strains of *Bifidobacterium*, namely *B. breve* ATCC 15700, *B. bifidum* ATCC 15696, *B. infantis* ATCC 15697, as well as *Lactobacillus acidophilus* CH-2, all of which were shown to contain Lf binding proteins within the membrane protein fraction while, conversely, no Lf binding proteins were detected in the membrane protein fraction of *B. longum* whose growth was not enriched in the presence of Lf (Kim et al. 2004). It should be noted, however, that Petchow *et al.* reported that while Lf binding may be involved, Lf exerts a bifidogenic effect *via* a mechanism which is independent of both iron saturation and receptor binding (Petschow et al. 1999). Multiple *in vivo* studies have also established that Lf exerts bifidogenic effects. Hentges *et al.* reported gnotobiotic mice associated with a human infant gut microbiota consuming a milk fortified with bovine Lf had increased counts of *Bifidobacterium* compared with mice consuming un-supplemented formula (Hentges et al. 1992). Furthermore, recombinant human Lf (rhLf) was shown by Hu *et al.* to modulate the intestinal microbiota of piglets and increase ileal populations of *Bifidobacterium spp.* and *Lactobacillus spp.* (Hu et al.

2012). A recent study by Mastromarino *et al.* demonstrated a correlation between the level of Lf present in breast milk and the proportions of *Lactobacillus* and *Bifidobacterium* present in the feces of new born babies (Mastromarino et al. 2014). Notably, poor maternal health is associated with variations in breast milk composition which include a reduced level of Lf (Hennart et al. 1991).

Ultimately, these combined results suggest that the ingestion of Lf or peptides resulting from its digestion can have prebiotic effects.

### **1.6.5 Lactoperoxidase**

A member of the mammalian peroxidases family, the lactoperoxidase (LP) enzyme accounts for 0.25-0.50% of the total protein content of whey and is found in biological secretions such as tears, saliva and milk. One of the most abundant enzymes in plain milk, LP plays an important role in its preservation and has several commercial applications such as food and crop preservation and protection of the oral cavity against bacterial infection (Reiter et al. 1963, Seifu et al. 2005, Boots and Floris 2006). The anti-bacterial activity of LP relies on the presence of both the thiocyanate anion ( $\text{SCN}^-$ ; which is present in significant levels in natural secretions such as saliva and milk) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and is based upon the ability of LP to catalyse the oxidation of the  $\text{SCN}^-$  (Reiter and Härnult 1984, Reiter and Perraudin 1991). This LP-catalysed reaction results in the formation of the short-life intermediary compounds, primarily hypothiocyanate ( $\text{OSCN}^-$ ) and hypothiocyanous acid ( $\text{HOSCN}$ ), which are thought to invoke anti-bacterial activity *via* the inhibition of essential bacterial metabolic pathways such as glycolysis by the oxidation of sulfhydryl groups of bacterial proteins in both Gram positive and Gram negative bacteria (Reiter and Härnult 1984, Reiter and Perraudin 1991, Tenovuo 2002). The

formation of intermediary compounds from the LP-catalysed oxidation of  $\text{SCN}^-$  is pH dependant and may result in the formation of other bactericidal compounds such as cyanosulphurous acid ( $\text{HO}_2\text{SCN}$ ) and cyanosulphuric acid ( $\text{HO}_3\text{SCN}$ ). However, at physiological pH,  $\text{OSCN}^-$  is the major oxidation product (Björck 1978, Pruitt and Tenovou 1982). The LP anti-bacterial system plays an important role in the maintenance of oral health, where the LP/ $\text{SCN}^-/\text{H}_2\text{O}_2$  system was shown by Welk *et al.* to have bactericidal activity against both *Streptococcus mutans* and *S. sanguinis*, which are associated with both dental caries and periodontal disease (Loesche 1986, Kolenbrander and London 1993, Welk et al. 2011).

## **1.7 Beneficial effects of dietary whey protein**

Bovine whey proteins have been the subject of much interest due to the beneficial effects which they have been shown to exert on a variety of physiological processes such as energy balance, appetite and glucose metabolism, all of which are relevant to the use of whey proteins in the treatment of diseases such as obesity and T2D. (Table 1).

### **1.7.1 Weight loss and body composition**

With the current worldwide obesity epidemic, the anti-obesity properties associated with whey proteins have attracted much attention in recent years. Several studies have highlighted the anti-obesity effects of whey proteins or, more specifically, their ability to reduce high fat diet associated increased body weight and fat mass accumulation. Shertzer *et al.* reported female C57BL/6J mice fed a high fat diet with 100g whey protein isolate (WPI)/L drinking water showed reduced body fat and body weight gain, while having increased lean mass, compared to their littermates which did not receive WPI supplemented drinking water (Shertzer et al. 2011).

Similarly, in another study using C57BL/6J, Shi *et al.* reported a dose dependant reduction in weight gain and the development of fatty liver upon replacing 5%, 50% or 100% of the dietary casein protein-derived energy with WPI enriched in LP and Lf (Shi et al. 2011). Pilvi *et al.* have suggested several mechanisms by which whey proteins may reduce symptoms of obesity in a series of studies in C57BL/6J mice consuming high fat diets. Initially, it was shown that mice in receipt of a diet consisting of 60% of total calories from fat, 18% from whey (WPI) and 1.8% calcium carbonate ( $\text{CaCO}_3$ ) showed a significant decrease in weight gain and body fat coupled with significantly higher levels of fat excretion when compared to mice in receipt of a similar diet where casein was the protein source and which had with a lower percentage of  $\text{CaCO}_3$  (0.4%). It was suggested that the reduction in weight gain and fat excretion results from the high leucine content of whey modulating insulin signalling, thus directing energy towards muscle biosynthesis rather than fat storage in adipose tissue (Pilvi et al. 2007). In a follow-up study employing the same diet groups for the same 21 week study period, the authors observed the same inhibition of fat mass accumulation within the whey consuming cohort combined with a significant up-regulation of leptin and  $\beta$ 3-adrenergic receptor genes, leading the authors to suggest that whey protein attenuates symptoms of obesity *via* improved leptin sensitivity and increased lipolysis (Pilvi et al. 2008). Finally, in a later study by the same group, mice with diet induced obesity were placed on a low-calorie weight loss diet containing different whey proteins ( $\alpha$ -La,  $\beta$ -lac, WPI and Lf) for 50 days before being returned to a high fat diet containing the same whey protein/WPI for a further 50 days. Mice which consumed the  $\alpha$ -La diet showed a significant loss of fat mass during the calorie restricted period and a subsequent reduction in visceral fat during the weight recovery period compared to all other



dietary groups (Pilvi et al. 2009). Pichon *et al.* suggested that  $\beta$ -lac is the most efficient whey protein in reducing weight gain after reporting a greater reduction in body weight and adipose tissue in rats consuming a high protein diet comprised of WPI enriched in  $\beta$ -lac than rats consuming a diet containing other protein sources such as whole milk and whey protein concentrate (WPC). Notably, however, this study did not include a diet enriched with  $\alpha$ -La (Pichon et al. 2008).

### **1.7.2 Appetite**

Another mechanism by which whey proteins may influence energy balance is through appetite control *via* their satiating properties. While it is generally accepted that dietary protein induces a greater satiating effect than other macronutrients such as carbohydrates or fats (Tomé et al. 2009), data from several studies have suggested that whey proteins are more potent satiety inducers than other protein sources. A study by Hall *et al.* reported that the intake of whey protein (48g) delayed the desire to consume a subsequent meal by 180 minutes when compared to casein in lean individuals (Hall et al. 2003). In another human study it was shown that the consumption of a diet of protein, carbohydrate and fat, which provided 10%, 55% and 35% energy, respectively, reduced hunger more effectively when whey protein was included in the diet rather than casein or soy protein (Veldhorst et al. 2009). In a further study in lean subjects, Pal and Ellis reported significantly reduced mean energy intake and reduced appetite during a subsequent buffet meal (4h later) in individuals who had consumed a blended drink containing whey protein, compared to those which had consumed a blended drink containing the same amount (50g) of either egg, tuna or turkey protein (Pal and Ellis 2010).

It has been suggested that whey provides a more satiating effect than other proteins due to its higher content of BCAAs and, in particular, leucine (Mastromarino et al. 2014). Leucine, which enters the brain more rapidly than any other amino acid (Yudkoff et al. 2005), has been shown through intracerebroventricular injection to play an important role in appetite suppression, which suggests that whey proteins invoke a central effect on energy intake (Morrison et al. 2007). It is also possible that these effects are mediated by the high tryptophan content in whey. As previously stated, tryptophan is a precursor to serotonin which is an important regulator of appetite (Ashley et al. 1979), thus the high tryptophan content of  $\alpha$ -La may in turn increase satiety through increased serotonin production. Regardless, a study by Veldhorst *et al.* demonstrated that a breakfast diet where  $\alpha$ -La provided either 10% or 25% energy reduced lunchtime energy intake when compared to a breakfast diet deriving protein energy from casein, soy or whey (Veldhorst et al. 2009). This is consistent with the observations of Hursel *et al.* who also reported that an  $\alpha$ -La enriched diet had a greater effect on appetite suppression than a whey diet (Hursel et al. 2010). These results suggest a use for  $\alpha$ -La in appetite control.

Another mechanism by which whey proteins (or whey peptides resulting from gastric digestion) may influence energy intake is by the stimulation of gastrointestinal hormones which are important regulators of energy intake (Pimentel and Zemdegs 2010). Cholecystokinin (CCK) is a hormone secreted by I cells of the small intestine whose stimulation results in increased satiety (Figlewicz et al. 1992). GMP was shown by Pederson *et al.* to stimulate the production of CCK in anaesthetised rats (Pedersen et al. 2000), thus suggesting a role for GMP in food intake regulation. Furthermore, results of the aforementioned Veldhorst *et al.* study support this suggestion in that they reported a greater satiety effect in subjects

consuming a breakfast diet comprising 10% whey protein compared to those consuming a breakfast diet comprising 10% whey protein without GMP (Veldhorst et al. 2009).

Other important gut hormones which influence energy intake are glucagon-like peptide-1 (GLP-1) and ghrelin, whose activation decrease and increase energy intake, respectively (Turton et al. 1996, Wren et al. 2001). In one study in which dietary whey protein intake (50g) decreased appetite in both lean and overweight individuals, it was found that this change was coupled with increased plasma levels of CCK and GLP-1 and reduced levels of ghrelin when compared to glucose intake (Bowen et al. 2006). The results of this study are in line with those of other studies in both human and animal models (Hall et al. 2003, Zhou et al. 2011). These results suggest that whey protein or their associated bioactive peptides can reduce energy intake by the activation or suppression of important appetite regulating hormones within the gut.

Notably, while the previously discussed results suggest a use for whey proteins in the development of treatments for obesity, some studies have suggested that the satiating effect of whey proteins may be reduced in obese individuals/animals. In a study using DIO mice, a similar energy intake pattern was observed in mice which had whey supplemented drinking water (100g/L) compared with those provided with un-supplemented drinking water, despite the former group having significantly lower body weights (Eller and Reimer 2010). This trend has also been shown in humans where whey protein supplementation reduced subsequent energy intake in normal weight but not obese individuals (Bellissimo et al. 2008). These observations are consistent with results reported in another human study by Bowen *et al.* (Bowen

et al. 2006). Results from these studies suggest that the beneficial effects of whey proteins on appetite suppression may be more potent in lean than obese individuals.

### **1.7.3 Glucose metabolism**

Dietary whey protein has been shown to exert insulinitropic and glucose lowering properties in several studies of healthy, insulin resistant and type-2-diabetic subjects. In a study of healthy individuals, Peterson *et al.* demonstrated that consumption of a single dose of carbohydrate (50g) supplemented with whey protein (20g) resulted in a significant reduction in postprandial blood glucose (Petersen et al. 2009). 12 weeks of whey protein supplementation was shown to decrease both fasting plasma insulin and insulin resistance in overweight subjects, suggesting that long term whey protein consumption improves insulin sensitivity (Pal et al. 2010). A significant hypoglycaemic effect was observed in insulin resistant subjects who consumed a drink containing 50g of glucose and 30g of whey compared with those who consumed a drink containing the same 50g of glucose with or without canola oil (Lan-Pidhainy and Wolever 2010). Furthermore, whey proteins have been shown to exert a greater insulinitropic effect than other dietary proteins, such as casein (Pal and Ellis 2010), gluten and codfish (Mortensen et al. 2009).

One proposed mechanism by which whey proteins may invoke insulinitropic effects is *via* the formation of whey-associated bioactive peptides which, in turn, act as endogenous inhibitors of dipeptidyl peptidase 4 (DPP-IV). DPP-IV is an antigenic enzyme whose principal *in vivo* function is the rapid degradation of the incretins GLP-1 and gastric inhibitory peptide (GIP) which induce insulin secretion (Kieffer et al. 1995, Holst and Gromada 2004). Gunnarsson *et al.* showed a significant

reduction in DPP-IV activity upon administration of whey protein in anaesthetised C57BL/6J mice (Gunnarsson et al. 2006), while other studies, using both healthy and diabetic subjects, have reported increases in both GIP and GLP-1 after whey protein supplementation (Hall et al. 2003, Ma et al. 2009, Veldhorst et al. 2009). The balance between these incretins and DPP-IV appears to be altered in T2D patients where increased DPP-IV activity and decreased postprandial GLP-1 levels have been reported (Mannucci et al. 2005, Petersen et al. 2009). These results therefore suggest a use for dietary whey proteins in non-pharmaceutical treatment for the improvement of insulin sensitivity and, potentially, in the treatment of diabetic patients.

### **1.8 Whey proteins and the gut microbiota**

The composition of the gut microbiota undergoes dramatic changes throughout the life cycle of the host. Component proteins of whey appear to play important roles in the development of the infant gut microbiota by stimulating beneficial bacteria species such as members of the *Bifidobacterium* and *Lactobacillus* genera and providing protection against opportunistic pathogens such as *E. coli* (Adlerberth and Wold 2009, Hascoët et al. 2011, Mastromarino et al. 2014, Subramanian et al. 2015). However, the impact of dietary whey protein intake on the stable adult gut microbiota is currently poorly understood. Some promising results have been reported in the few studies examining the effect of dietary whey protein on the gut microbiota. Firstly, as noted above, when compared to other dietary protein sources such as red and white meat, soya and casein, whey proteins were shown to exert significantly less colonocyte DNA damage in rats (Toden et al. 2007, Toden et al. 2007). This result suggests that bacterial fermentation of whey proteins does not result in a large array of putrefactive metabolites traditionally associated with high

dietary protein intake, and therefore whey proteins represent potential novel candidates for beneficial gut microbiota modulation. Sprong *et al.* reported that consumption of diet containing cheese whey protein increased in fecal *Lactobacillus* and *Bifidobacterium* counts in Wistar rats when compared to those consuming the same diet but where the protein energy source was casein (Sprong et al. 2010). Similar results were reported by McAllen *et al.* who, using 16S rRNA sequencing, demonstrated significantly higher *Lactobacillus* and *Bifidobacterium* populations with a concurrent significant decrease in *Clostridium* populations in C57BL/6 mice consuming a high fat diet containing WPI (20% kJ) compared to mice consuming a high fat diet containing casein (20% kJ) (McAllan et al. 2014). However, in a similar study, Tranberg *et al.* did not observe any difference in the composition of the gut microbiota between two groups of C57BL/6 mice consuming a high fat diet where the protein source was either casein or whey, although in this case microbiota analysis was determined by less detailed denaturant gradient gel electrophoresis (DGGE) (Tranberg et al. 2013). Notably, a recent study showed that, in comparison to a standard diet, an enteral whey-peptide based murine diet significantly increased concentrations of cecal SCFAs. While results from this study indicate that the microbial fermentation of whey proteins or associated bioactive peptides promote gut health through increased SCFA production, the authors did not examine the composition of the gut microbiota and thus the effect that whey proteins have on SCFA producing bacterial species was not ascertained (Smith et al. 2013, Tomoda et al. 2015). In our own recent study, the effect of replacing casein (20%) as the protein source in a high fat murine diet with the whey proteins Lf and BSA on the composition of the gut microbiota was examined over an eight week intervention period. High-throughput sequencing of the murine fecal microbiota revealed that

both BSA and Lf reversed several of the high fat associated alterations in microbial taxonomy and, in the case of Lf, significantly enriched the proportions of the *Roseburia* genus (Chapter 3).

Finally, Clarke *et al.* observed a significantly higher microbial diversity, which as noted earlier is a biomarker of good gut health associated with the gut of professional rugby players relative to controls. This higher diversity was positively correlated with protein intake with, notably, whey protein supplements representing a significant proportion of the athlete's total protein consumption (Clarke *et al.* 2014). While data from these initial studies suggest a potential use for whey proteins in the beneficial modulation of the gut microbiota, more studies using specific component proteins of whey are required to fully elucidate their effects on enteric bacterial populations.

## **1.9 Conclusions**

It is now well established that the gut microbiota plays a pivotal role in maintaining human health and that imbalances in the complex microbial communities residing within the gut can have serious health implications in several GI disease states. Given that the current strategies employed to prevent/reduce the growing trend of inflammatory diseases such as obesity have been largely unsuccessful (Roberto *et al.* 2015), selective modulation enteric microbial communities is an area which has the potential to deliver novel and effect treatments for such diseases (Walsh *et al.* 2014). To date, efforts to selectively modulate these microbial communities using dietary components have been restricted to non-digestible carbohydrates which have been shown to stimulate the growth of beneficial bacterial species and improve disease severity (Manning and Gibson 2004, Dehghan *et al.* 2014), while dietary protein has

received less attention and been associated with the formation of putrefactive bacterial metabolites (Louis et al. 2014). Dairy whey proteins have been shown to exert beneficial effects of a variety of physiological processes, such as energy balance and glucose metabolism, however their impact on gut microbial populations is less well known. Similar to prebiotic intervention studies, preliminary studies examining the effect of dairy whey protein on the gut microbiota, have shown whey protein consumption to enrich populations of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Sprong et al. 2010, McAllan et al. 2014). Notably whey protein consumption was shown to exert significantly less colonocyte DNA damage than other dietary protein sources (Toden et al. 2007) suggesting that whey proteins may beneficially modulate the gut microbiota without the formation of putrefactive metabolites traditionally associated with proteolytic bacterial fermentation. These primarily studies suggest that whey proteins may represent novel agents to beneficially modulate the gut microbiota however.



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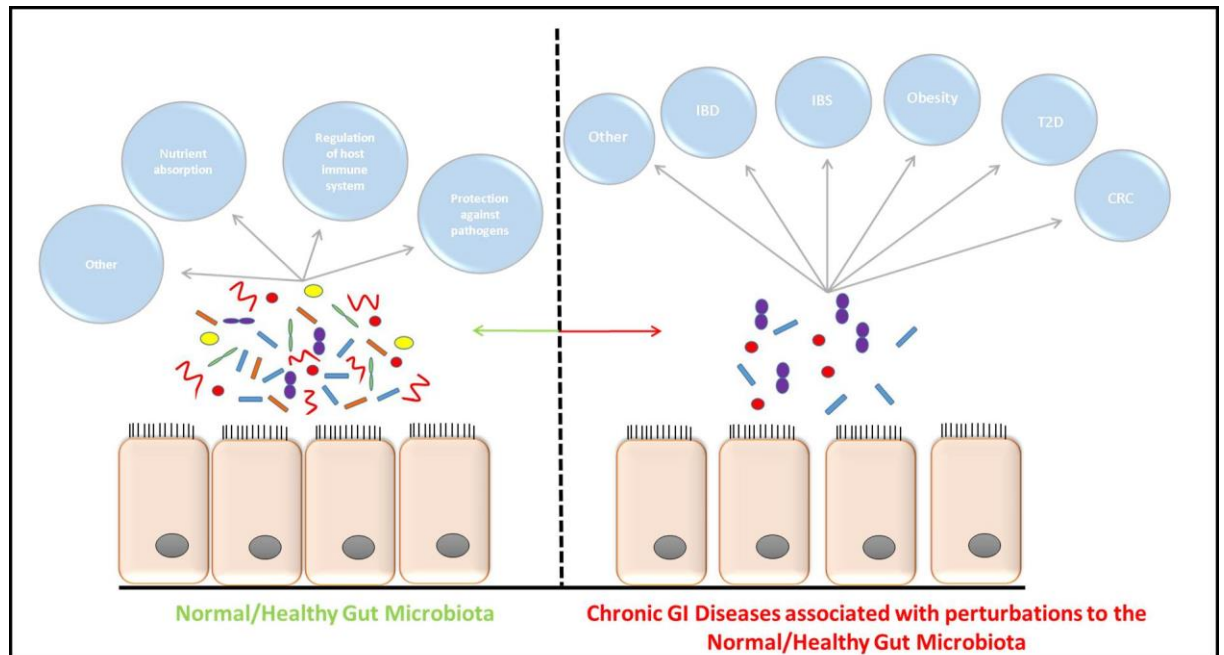
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**Table 1. Bioactive properties of bovine whey proteins.**

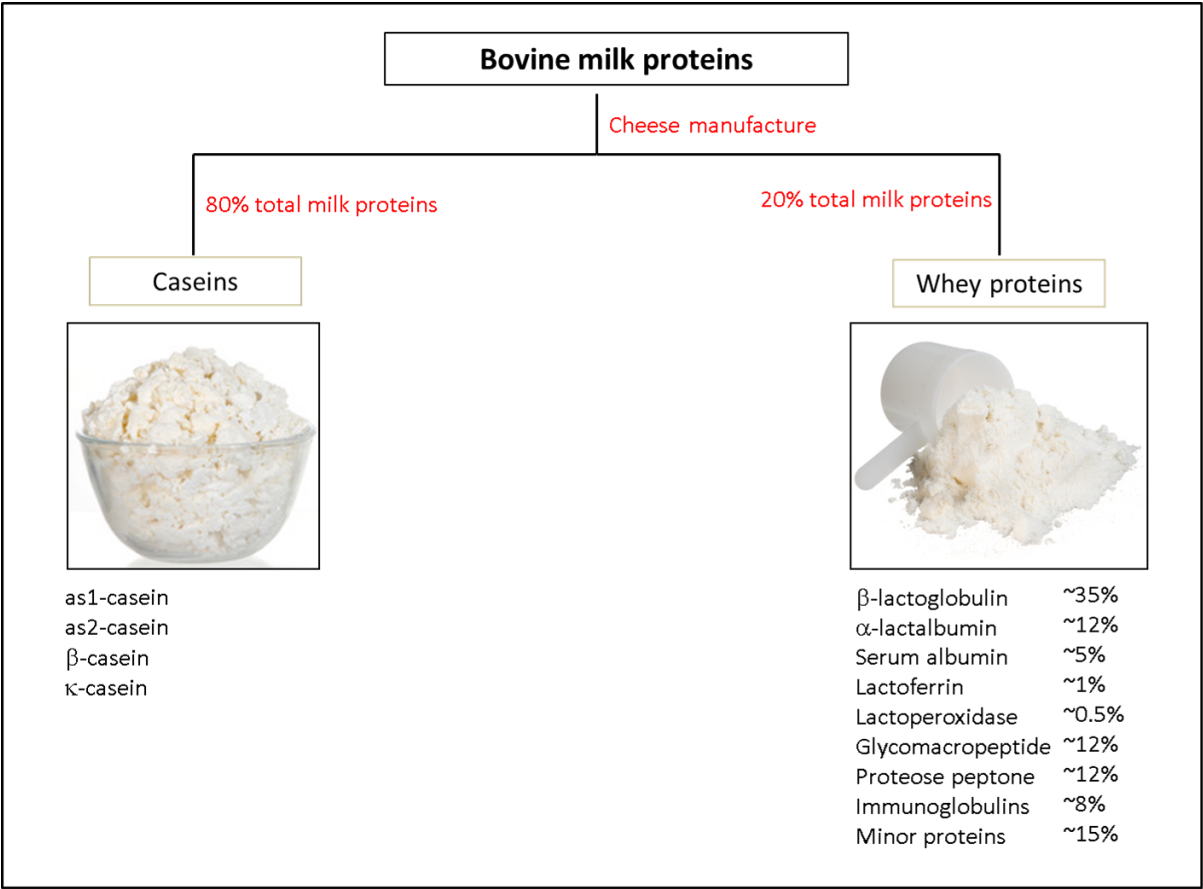
Whey component Protein	AA residues	Associated Bioactive properties	References
<b>β-lactoglobulin</b>	162	Intestinal uptake of retinol, triglycerides and long chain fatty acids.	(Kushibiki et al. 2001)
		Delivery vehicle for prebiotic compounds	(Livney 2010, Kent and Doherty 2014)
		Bactericidal activity (upon trypsin digestion) against Gram positive bacteria	(Pellegrini et al. 1999)
<b>α-lactalbumin</b>	123	Co-enzyme in lactose production	(De Wit 1998)
		Inhibition of tumour growth and proliferation	(Sternhagen and Allen 2001, Rammer et al. 2010)
		Improvements in mood and cognition due to high tryptophan content	(Orosco et al. 2004, Markus et al. 2005)
<b>Bovine Serum Albumin</b>	582	Maintenance of colloid osmotic pressure	(Hankins 2006)
		Carrier protein for catecholamines, free fatty acids and other hormones	(Danon and Sapira 1972)
		Inhibition of tumour cells in MCF-7 human breast cancer cell lines	(Laursen et al. 1989)
<b>Lactoferrin</b>	700	Reduction of fat mass accumulation	(McManus et al. 2015)
		Anti-viral	(Van der Strate et al. 2001)
		Anti-carcinogenic	(Hayes et al. 2006, Nakajima et al. 2011)
		Anti-inflammatory	(Conneely 2001)
		Bacteriostatic activity <i>via</i> iron sequestration	(Arnold et al. 1980, Reyes et al. 2005)
		Bactericidal activity against a wide range of Gram positive and Gram negative bacteria	(Ellison et al. 1988, Appelmek et al. 1994)
		Increased potency of other antibacterial agents	(Kawasaki et al. 2000, Ochoa et al. 2003, Ochoa and Cleary 2009)
		Release of potent antimicrobial peptides upon gastric digestion	(Sanchez and Watts 1999)
<b>Lactoperoxidase</b>	612	Release of potent antimicrobial peptides upon gastric digestion	(Bellamy et al. 1992, Vorland et al. 1998)
		Growth promotion of <i>Bifidobacterium</i> species	(Yamauchi et al. 1993, Vorland et al. 1999)
		Preservation of plain milk	(Saito et al. 1996, Rahman et al. 2010)
<b>Lactoperoxidase</b>	612	Protection of the oral cavity against bacterial infection	(Mastromarino et al. 2014)
			(Seifu et al. 2005)
<b>Lactoperoxidase</b>	612		(Boots and Floris 2006, Welk et al. 2011)

**Figure 1. Gut microbiota in health and disease.** Diagram representing some of the health benefits conferred by the 'normal/functioning' gut microbiota (left) and chronic GI diseases associated with perturbations to the gut microbiota (right).





**Figure 2. Bovine milk protein system.**



## **Chapter 2**

**Protein quality and the protein to carbohydrate ratio within a high fat diet influences energy balance and the gut microbiota in C57BL/6J mice.**

### **Peter Skuse Chapter Contributions**

#### **Experimental:**

- Designed and performed all experiments relating to fecal sample DNA extraction, PCR amplification and 454-pyrosequencing.

#### **Results interpretation:**

- Analysis of all data pertaining to 16S rRNA gene sequencing.

#### **Manuscript preparation:**

- Major contributor to manuscript preparation.

**PloS one 9.2 (2014): e88904**

## 2.1 ABSTRACT

Macronutrient quality and composition are important determinants of energy balance and the gut microbiota. Here, we investigated how changes to protein quality (casein versus whey protein isolate; WPI) and the protein to carbohydrate (P/C) ratio within a high fat diet (HFD) impacts on these parameters. Mice were fed a low fat diet (LFD)(10% kJ) or a high fat diet (HFD; 45% kJ) for 21 weeks with either casein (20% kJ, HFD) or WPI at 20%, 30% or 40% kJ. In comparison to casein, WPI at a similar energy content normalised energy intake, increased lean mass and caused a trend towards a reduction in fat mass ( $P=0.08$ ), but the protein challenge did not alter oxygen consumption or locomotor activity. WPI reduced HFD-induced plasma leptin and liver triacylglycerol, and partially attenuated the reduction in adipose FASN mRNA in HFD-fed mice. High throughput sequence-based analysis of faecal microbial populations revealed microbiota in the HFD-20% WPI group clustering closely with HFD controls, although WPI specifically increased *Lactobacillaceae/Lactobacillus* and decreased *Clostridiaceae/Clostridium* in HFD-fed mice. There was no effect of increasing the P/C ratio on energy intake, but the highest ratio reduced HFD-induced weight gain, fat mass and plasma triacylglycerol, non-esterified fatty acids, glucose and leptin levels, while it increased lean mass and oxygen consumption. Similar effects were observed on adipose mRNA expression, where the highest ratio reduced HFD-associated expression of UCP-2, TNF $\alpha$  and CD68 and increased the diet-associated expression of  $\beta$ 3-AR, LPL, IR, IRS-1 and GLUT4. The P/C ratio also impacted on gut microbiota, with populations in the 30/40% WPI groups clustering together and away from the 20% WPI group. Taken together, our data show that increasing the P/C ratio has a dramatic effect on energy

balance and the composition of gut microbiota, which is distinct from that caused by changes to protein quality.

## 2.2 INTRODUCTION

It is widely recognised that levels of obesity and related clinical conditions such as diabetes, stroke, hyperlipidemia and cardiovascular disease are increasing worldwide (Caterson et al. 2004). Importantly, the development of obesity increases the set point at which the body weight, more specifically body fat, is defended, thus making its reversal difficult to achieve (Guo et al. 2009, Ryan et al. 2012). As such, there is an increased research interest to develop effective treatments for this disease.

Dairy proteins belonging to the whey fraction (a by-product of cheese manufacture) have been increasingly tested for their potential anti-obesity effect, specifically for their ability to reduce high fat diet (HFD)-associated body weight and fat mass gain (Pilvi et al. 2007, Shertzer et al. 2011, Shi et al. 2011). Shi *et al.*, (2012) showed that replacing 5%, 50% or 100% of the dietary casein protein-derived energy content with a lactoperoxidase and lactoferrin-enriched whey protein isolate (WPI) caused a proportional suppression of body weight gain in HFD fed mice. We have previously demonstrated that a WPI-related reduction in body weight and fat mass gain in HFD fed mice was accompanied by a normalisation of energy intake and complete or partial reversal of energy balance-related gene expression in the adipose tissue and the hypothalamus (McAllan et al. 2013). While these data suggest that whey proteins have a specific-effects on energy balance, such effects appear be modified by the macronutrient composition in the diet (Pichon et al. 2008). In the latter study, it was shown that increasing the lipid to carbohydrate ratio within a whey protein-rich diet significantly reduced energy intake and bodyweight gain in rats. Collectively, these data suggest that protein quality and macronutrient composition are important determinants of energy balance.

Interestingly, diet is also an important factor in determining the composition of the gut microbiota (Turnbaugh et al. 2009, Wu et al. 2011) and specific gut microbiota signatures are associated with obesity phenotypes in animals and humans (Backhed et al. 2004, Turnbaugh et al. 2006, Murphy et al. 2010). Notably, studies have shown specific whey proteins to possess anti-microbial activity (Yamauchi et al. 1993, Freedman et al. 1998, Shin et al. 2000), and that the digestive process itself facilitates the formation of potent antimicrobial whey-derived peptides, such as pepsin catalysed lactoferrin to lactoferricin (Hoek et al. 1997). A study by Sprong *et al.*, (Sprong et al. 2010) demonstrated that in comparison to casein, whey protein intake increased levels of lactobacilli and bifidobacteria in a rat model of colitis. However, in a more recent study, whey protein intake was found to have no influence on gut microbiota composition in mice fed a HFD for 7 or 13 weeks (Tranberg et al. 2013). Several key unanswered questions are; could whey proteins specifically influence the gut microbiota composition associated with prolonged high fat feeding, and would any changes relate to energy balance? Could changes to protein to carbohydrate ratio within a HFD vary the gut microbiota profile and energy balance in a different way to changes to protein quality?

To assess WPI specific effects on above parameters, we subjected male C57BL/6J mice to 21 weeks of either a low fat diet (LFD) with 20% kJ casein or a HFD with 20% kJ casein or WPI. In addition, using two additional HFD dietary groups on 30 or 40% kJ WPI, we evaluated the impact of increasing the protein to carbohydrate (P/C) ratio within the HFD on parameters of interest. Our data show that WPI has a specific effect on energy balance and gut microbiota, while increasing the P/C ratio within the HFD leads to dramatic alterations in energy

balance, body composition, metabolic health and the composition of the gut microbiota.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 Ethics Statement**

All research involving mice was licensed under the Cruelty to Animal Act 1876 and received ethical approval from the University College Cork Animal Ethics Review Committee (#2011/005).

### **2.3.2 Animals**

Male 3-4 week old C57BL/6J mice (Harlan, Oxon, UK) were group housed either 5 per cage (Study 1) or 4 per cage (Study 2) in individually ventilated cages and acclimatised for four weeks in a light (06:00-18:00), temperature ( $21 \pm 1^{\circ}\text{C}$ ) and humidity (45-65%) controlled environment with free access to water and a low fat diet (LFD; 10% kJ fat and 20% kJ casein; #D12450, Research diets; New Brunswick, NJ, USA).

### **2.3.3 Experimental protocol**

Two studies were performed to assess how the WPI-derived energy content within a HFD (study 1) or LFD (study 2) impacts on energy balance-related parameters in mice over a 21 week (study 1) or 7 week period (study 2).

Study 1: Following the acclimatisation period, weight-matched dietary groups were maintained on the LFD or switched to either a HFD (45% kJ fat and 20% kJ casein; #D12451) or a HFD with WPI (Alacen<sup>tm</sup> 895 NZMP, New Zealand) at an energy content of 20% kJ (HFD-20% WPI), 30% kJ (HFD-30% WPI) or 40% kJ (HFD-40% WPI) (Supplementary table 1) (n = 10) for a total of 21 weeks. Body weights were measured weekly. Energy intake in group housed mice was measured by weighing the food hopper each week until week 16. During weeks 17-20, energy intake and metabolic activity in individual mice was measured using TSE Phenomaster cages (TSE systems, Bad Homburg, Germany). Following this analysis



and prior to re-housing the mice in home cages, faecal pellets were collected from individual mice for examination of microbial composition via pyrosequencing and subsequent bioinformatic analysis. At the end of the experimental period, mice were fasted for 6 hours and the body composition was measured using the Bruker minispec LF50H (Bruker optics, Ettlingen, Germany). Mice were then anaesthetised using ketamine (65mg/kg bodyweight) and xylazine (13mg/kg bodyweight). Blood was collected from anaesthetised mice into vacutainer EDTA tubes (BD, USA) and treated with Aprotinin (500,000 KIU/L final concentration; Sigma, Ireland) and Diprotin A (0.1mM final concentration; Sigma, Ireland) to protect plasma peptides from proteolytic degradation. Plasma was isolated from blood by centrifugation at 2000rpm at 4°C for 15 mins. Mice were sacrificed by cervical dislocation, and tissues of interest were dissected and snap frozen in liquid nitrogen (liver, adipose and stomach) or on dry ice (brain). Plasma and tissue samples were stored at -80°C until analysis.

Study 2: Weight matched mice were provided for 7 weeks with either the LFD or a LFD with WPI replacing the casein protein (LFD-WPI; 10% kJ fat and 20% kJ WPI) (n = 8). Body weights were measured weekly. Energy intake and metabolic activity in individual mice was measured during weeks 5 and 6 using the TSE Phenomaster system. After the analysis, mice were re-housed, as before, in the home cages and the experiment was terminated at the end of week 7.

#### **2.3.4 Analysis of metabolic parameters**

The TSE Phenomaster cages comprised an open-circuit indirect calorimetry system with gas sensing units to measure oxygen consumption (ml/h/kg) ( $\text{VO}_2$ ) and  $\text{CO}_2$  production (ml/h/kg) ( $\text{VCO}_2$ ). The cages also contained high precision sensor associated-feeding baskets to accurately measure food intake (g), with a meal

defined as intake over 0.01g. A multi-dimensional infrared beam system allowed the measurement of locomotor activity, which was defined as the total number of infrared beam breaks in the X and Y axis. Mice were singly housed in TSE Phenomaster cages for a total of 3 days, with data collected during the final 24 hours, following a 2 day acclimatisation to the new cage environment. The acclimatisation period was established based on the data from our previous study (McAllan et al. 2013). Heat production (kcal/h/kg) in individual mice was calculated using the Weir equation ( $3.941 \times \text{VO}_2 + 1.106 \times \text{VCO}_2$ )(Weir 1949)), and this was converted to kJ/h/kg using  $1\text{kcal} = 4.184\text{ kJ}$ . The respiratory exchange ratio (RER) was calculated by  $\text{VCO}_2/\text{VO}_2$ . Energy intake was calculated from food intake measurements using the energy content of the diets supplied by the manufacturer.

### **2.3.5 Microbial DNA extraction, amplification and high throughput DNA sequencing**

Total metagenomic DNA was extracted from individual faecal samples using QIamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), after an additional bead-beating step. Bacterial composition was determined by sequencing of 16S rRNA amplicons (V4-V5 region; 408nt long) generated by a separate PCR reaction for each sample (in triplicate) using universal 16S primers, where, the forward primer (5'-AYTG GGYDTAAAGNG), with attached molecular identifier tags between 454 adapter sequence and target-specific primer sequence, and reverse primer V5 (5'-CCGTCAATTYYTTTTRAGTTT) (Claesson et al. 2010), were used along with Biomix Red (Bioline, London UK). The template DNA was amplified under the following PCR conditions for a total of 35 cycles: 94°C for 2 minutes and 1 minute respectively (initialization and denaturation), 56°C for 60 seconds (annealing) and 72°C for 60 seconds (elongation), proceeded by a final elongation stage of 2

minutes. Negative control reactions with PCR grade water in place of template DNA were used to confirm a lack of contamination. Amplicons were pooled and cleaned using the AMPure XP purification system (Beckman and Coulter, Takeley, UK) and DNA concentration was determined using the NANODROP 3300 Fluorospectrometer (Thermo Scientific, USA) coupled with the Quant-it™ Picogreen® dsDNA Assay Kit (Invitrogen, Paisley, UK). Equal volumes of each sample were then pooled together and underwent a final cleaning and quantification stage. Amplicons were sequenced in-house on a Roche GS FLX Titanium platform.

### **2.3.6 Bioinformatics**

Raw sequencing reads were ‘de-noised’ using traditional techniques implemented in the Ribosomal Database Project Pyrosequencing (RDP) Pipeline with ambiguous bases, non exact primer matches and reads shorter than 150bp being excluded. Trimmed FASTA files were then BLASTed against a previously published 16S-specific database using default parameters. The resulting files were then parsed using the MEGAN software package, which assigns reads to the National Centre for Biotechnology Information (NCBI) taxonomies via the lowest common ancestor algorithm. Results were filtered prior to tree construction and summarization by the use of bit scores from within MEGAN where a cut-off bit score of 86 was employed (Urich et al. 2008, Rea et al. 2011). The QIIME software suite was employed to achieve clustering of sequence reads into operational taxonomic units (OTUs) (Caporaso et al. 2010). Chimeric OTUs were removed using the ChimeraSlayer program (Haas et al. 2011) and phylogenetic trees constructed using the FastTreeMP tool (Price et al. 2010). Beta diversity values were calculated based on Bray Curtis, weighted and unweighted UniFrac distances, and the KING viewer was used to visualise resulting PCoA plots (Huson et al. 2007, Chen et al. 2009). Sequence reads

were deposited in the European Nucleotide Archive (EHA) under the accession number PRJEB4636.

### **2.3.7 Plasma analysis**

Colorimetric assays were used to measure plasma levels of glucose (Calbochem, Darmstadt, Germany), triacylglycerol (TAG; Wako Chemicals, Richmond, VI, USA) and non-esterified fatty acids (NEFA; Abcam, Cambridge, UK). Commercially available ELISA kits were used to analyse plasma levels of insulin, leptin (Crystal Chem, Downers Grove, IL, USA), glucagon-like peptide 1 (GLP-1; Millipore, St. Charles, MO, USA) and corticosterone (Enzo Life sciences, Farmingdale, NY, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was determined using the formula: fasting plasma insulin ( $\mu\text{U/ml}$ )  $\times$  fasting plasma glucose ( $\text{mmol/L}$ )/ 22.5 (Matthews et al. 1985). To measure plasma amino acid levels, samples were first deproteinised by mixing with equal volumes of 24% (w/v) tri-chloroacetic acid. The samples were then allowed to stand for 10 minutes before been centrifuged at 14400 x g (Microcentaur, MSE, UK) for 10 minutes. Supernatants were mixed with 0.2 M sodium citrate buffer, pH 2.2, and the plasma concentration of amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, UK) fitted with a Jeol  $\text{Na}^+$  high performance cation exchange column.

### **2.3.8 Liver TAG analysis**

Total lipids from liver samples (approx. 50mg) were extracted as described previously (McAllan et al. 2013) using the Folch extraction method (Folch et al. 1957). Briefly, total lipids were extracted using 2:1 (v/v) chloroform: methanol solution, into which a 0.88% NaCl solution was added before centrifugation at 2000rpm and 4°C for 30mins. Aliquots of the organic phase were collected, dried

and re-suspended in the LabAssay TAG reagent (Wako Chemicals, Richmond, VI, USA) to measure TAG levels using LabAssay TAG kit according the manufacturer's protocol.

### **2.3.9 Real-Time PCR analysis**

Total RNA was isolated from tissues using RNeasy mini (liver and stomach) or RNeasy lipid mini (adipose and hypothalamus) kits (Qiagen, Hilden, Germany) according to manufacturers' instructions. RNA was treated with DNase (Qiagen, Hilden, Germany) during RNA isolation to eliminate any possible genomic DNA contamination. Reverse transcription of 1µg of RNA was performed using 2.5ng/µl random hexamer primers (Bioline, London, UK), 0.5mM dNTP (Promega, Madison, VI, USA), 2U/µl RNase inhibitor (Promega, Madison, VI, USA), and the Superscript II first stand system (Invitrogen, Carlsbad, CA, USA) according to manufactures' instructions. Gene expression was measured by the Roche Lightcycler 480 system (Rotkreuz, Switzerland) via amplification of 1µl complementary DNA using the Lightcycler SYBR Green I Mastermix kit (Roche, Penzberg, Germany) and 2.5µM gene specific primers (Eurofins MWG operon, Ebersberg, Germany) in a 10µl total reaction volume. Primer sequences used are listed in supplementary table 2. PCR conditions were; 10mins at 95°C, followed by 50 cycles of 95°C for 10s, 58-65°C for 5s and 72°C for 15s. Authenticity of PCR products was determined by melting curve analysis and by automated sequencing. Crossing point (Cp) of fluorescence signals were used to calculate target gene expression by  $2^{-\Delta\Delta C_p}$ , following normalisation against housekeeping gene according to  $\Delta\Delta C_p = \Delta C_p \text{ target gene} - \Delta C_p \text{ housekeeping gene}$ . Housekeeping genes used were  $\beta$ -actin (liver, stomach and hypothalamus), YWHAZ (liver and hypothalamus), 18-S (adipose) and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (adipose and stomach). Relative gene expression is shown compared to the LFD group.

### **2.3.10 Statistical analysis**

Data are expressed as means  $\pm$  standard error of the mean (SEM). Differences between experimental dietary groups were analysed by one-way or two-way ANOVA followed by pairwise comparisons using *tukey* or *bonferroni* post hoc tests respectively. Body weight trajectories were analysed by two-way repeated measures ANOVA with *bonferroni* post hoc tests. Non-parametric data was compared by Kruskal-Wallis ANOVA followed by *Dunn's* pairwise comparisons. Significance levels were set at  $P \leq 0.05$ , and statistical analysis was performed using Graphpad prism (ver. 5.04; San Diego, CA, USA) and Minitab (ver.15; State College, PA, USA).

## 2.4 RESULTS

### 2.4.1 WPI inclusion or increasing the P/C ratio within a HFD alters body composition and plasma amino acids

Fig. 1A-B demonstrates that body weight gain of HFD-20%WPI fed mice was similar to HFD controls. However, intake of WPI was seen to have a specific effect on body composition, with HFD-20% WPI fed mice having an increased lean mass (%) ( $P < 0.05$ ), and a trend towards a reduction in fat mass (%) ( $P = 0.08$ ) compared to the HFD control group. Increasing the WPI derived energy content in the HFD to 40% and proportionally reducing the carbohydrate energy content led to a significant reduction in body weight gain compared to all other HFD-WPI groups ( $P < 0.001$ ), with observed values similar to that seen for the LFD group. This was accompanied by significantly reduced body fat mass and increased lean mass levels in the HFD-40% WPI fed mice compared to HFD control and other WPI diets groups ( $P < 0.001$ ), while the body composition of HFD-30% WPI group did not differ from that of the HFD-20% WPI group (Fig. 1C).

Comparison of the plasma amino acid profiles including those that could influence lean and fat mass, revealed an impact of WPI and the P/C ratio (Table 1). WPI specific effects were observed on glutamic acid, aspartic acid and glycine, which either decreased (glutamic acid and aspartic acid) or increased (glycine) compared to HFD fed mice ( $P < 0.05$ ) (Table 1). Changes in macronutrient ratio in HFD-40%WPI, decreased plasma histidine, phenylalanine, serine and threonine levels compared to the lowest P/C ratio (20% WPI) ( $P < 0.01$ ) (Table 1).

#### **2.4.2 WPI-enriched HFD normalised energy intake, while increasing the P/C ratio accentuated metabolism**

The cumulative energy intake (MJ) for the dietary groups (2 cages/group, all with  $n=5$  mice), measured over the first 16 weeks did not differ between LFD, HFD and HFD-20% WPI groups ( $24.73 \pm 2.70$  vs.  $27.69 \pm 1.54$  vs.  $29.40 \pm 0.31$  respectively). In contrast data gathered by individually housing the mice in TSE Phenomaster cages in week 17-20 demonstrated that the energy intake of the HFD-WPI groups was greater than that of the HFD control group during both the light and dark phases ( $P < 0.05$ ), while being similar to that of the LFD group (Fig. 2A). Increasing the P/C ratio had no significant effect on cumulative energy intake (MJ) in HFD fed mice (20% WPI,  $29.40 \pm 0.31$  vs. 30% WPI,  $30.01 \pm 0.62$  vs. 40% WPI,  $26.71 \pm 0.15$ ). Energy intake measurements from TSE Phenomaster cages corroborated this data (Fig 2A). There was also no significant effect on meal number or meal size of altering the P/C ratio (i.e. between WPI groups; Fig 2B-C).

The HFD-20% WPI diet had no impact on  $\text{VO}_2$ , heat production, locomotor activity or respiratory exchange ratio (RER) when compared to HFD fed mice (Fig. 3A-D) Increasing the P/C ratio was found to impact on energy expenditure with HFD-40% WPI fed mice having significantly increased levels of dark phase  $\text{VO}_2$  compared to HFD-20 and 30% WPI fed mice ( $P < 0.001$ ) (Fig 3A). A similar change in heat production was observed between the groups, albeit data was only significant between HFD-40% and HFD-30% WPI groups ( $P < 0.05$ ) (Fig 3B). There was no effect of WPI or P/C ratio on locomotor activity (Fig 3C). RER values of all HFD groups were lower than the LFD group in both the light and dark phases, consistent with increased fat metabolism ( $P < 0.001$ ) (Fig 3D).



Investigation of the above parameters in mice fed a LFD with WPI or casein for 7 weeks (study 2) revealed that WPI does not influence body weight, energy intake,  $\text{VO}_2$ , locomotor activity or RER in a low fat background (Supplementary Figure 1A-D).

### **2.4.3 Increasing the protein to carbohydrate ratio attenuated adverse metabolic impact of HFD**

Specific effects of WPI and the P/C ratio were observed on lipid metabolism-related gene expression and on tissue lipid deposition. Firstly, the decrease in epididymal adipose tissue fatty acid synthase (FASN) mRNA expression with HFD feeding, was somewhat attenuated by WPI challenge ( $P < 0.05$ ), with no added benefit of increasing the P/C ratio on expression of this gene (Fig 4A). Notably, the epididymal mRNA expression of a number of other genes were altered by the P/C ratio, specifically, fatty acid transporter 1 (FATP1), beta-3 adrenergic receptor ( $\beta 3$ -AR), peroxisome proliferator-activated receptor gamma ( $\text{PPAR}\gamma$ ), uncoupling protein 2 (UCP-2) and lipoprotein lipase (LPL) ( $P \leq 0.05$ ) (Fig 4). In the liver, WPI specifically reduced TAG levels (Table 2) and the mRNA expression of fatty acid binding protein 1 (FABP1) compared to HFD fed mice (Table 3). The highest P/C ratio (40% WPI) significantly decreased mRNA levels of cluster of differentiation 36 (CD36) and  $\text{PPAR}\gamma$  group ( $P < 0.05$ ) (Table 3), dramatically reduced liver TAG levels compared to 20/30% WPI fed mice (Table 2), and normalised the elevated plasma levels of TAG and NEFA observed with HFD feeding ( $P \leq 0.05$ ) (Table 2). Finally, the mRNA level of lipid metabolism-related carnitine palmitoyltransferase 1a-c (CPT1a-c), fatty acid transport protein 5 (FATP5) and  $\text{PPAR}\alpha$ , in tissues of interest, was not influenced by the dietary challenges (Tables 3-4).

Increasing the P/C ratio reduced plasma glucose levels, particularly in the 40% WPI group ( $P < 0.05$ ) (Table 2). In parallel HOMA-IR values were also reduced ( $P < 0.05$ ), but the change in plasma insulin concentration did not reach statistical significance (Table 2). At a cellular level, the highest P/C ratio normalised the HFD-induced reduction in adipose expression of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1), and partially prevented the HFD-induced reduction in glucose transporter 4 (GLUT4) (Fig. 4B) ( $P < 0.001$ ). In the hypothalamus, WPI specifically increased IR mRNA expression ( $P < 0.05$ ), as did P/C ratio, with the highest P/C ratio having the greatest impact (Table 4). Epididymal adipose tissue mRNA expression of inflammatory markers, namely tumour necrosis factor (TNF)- $\alpha$  and cluster of differentiation (CD) 68 only responded to the highest P/C ratio, which significantly reduced the expression of both in a HF background (Fig. 4B) ( $P < 0.001$ ). In the hypothalamus, whilst TNF $\alpha$  mRNA was elevated by HFD feeding, neither WPI nor the P/C ratio influenced its levels, although there was a trend towards a decrease for the highest P/C ratio (40% WPI) (Table 4). None of the dietary challenges influenced hepatic glucose transporter 2 (GLUT2), IRS-1 and TNF- $\alpha$  mRNA expression (Table 3) or hypothalamic IRS-1 mRNA expression (Table 4).

The increased plasma leptin concentration in response to the HFD was significantly blunted by WPI intake with dramatic reductions seen at the highest P/C ratio ( $P < 0.001$ ) (Table 2). Yet, the hypothalamic expression of genes known to be responsive to plasma leptin levels were unaffected, specifically mRNA levels of leptin receptor (ObR), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and growth hormone secretagogue receptor (GHS-R) (Table 4). In addition, gastric mRNA expression for the orexigenic hormone ghrelin was not found to significantly

differ between all dietary treatment groups ( $1.00 \pm 0.37$ , LFD vs.  $1.11 \pm 0.40$ , HFD vs.  $0.73 \pm 0.35$ , 20%-WPI vs.  $0.29 \pm 0.28$ , 30%-WPI vs.  $1.07 \pm 0.45$ , 40%-WPI). Plasma corticosterone levels were also elevated with HF feeding, but were not influenced by protein source (WPI or casein) or P/C ratio (Table 2). Similarly, there was no effect of WPI on the HFD-associated suppression of adipose  $11\beta$ -hydroxysteroid dehydrogenase type 1 ( $11\beta$ HSD1) (Fig 4B), or the HFD-induced increase in glucocorticoid receptor (GCCR) in the hypothalamus (Table 4).

#### **2.4.4 WPI inclusion or increasing the P/C ratio within a HFD altered the composition of gut microbiota**

A total of 251,395 V4-V5 16S rRNA sequence reads were generated which corresponded to an average of 50,279 reads per diet group or 5,130 reads per animal.  $\alpha$ -diversity values were calculated for biodiversity (Shannon index), species richness (Chao1) and the number of species relative to the abundance in the sample (Simpson diversity index). When  $\alpha$ -diversity values were compared by diet group, the only difference observed was a significantly higher microbial richness (Chao1) within the HFD microbiota compared to the HFD-30% WPI ( $P = 0.028$ ). Principal coordinate analysis (based on unweighted UniFrac distances) (Fig. 5) of the sequence data highlighted a clustering of the LFD, HFD and HFD-20% WPI group microbial populations, while HFD-30% and 40% WPI groups clustered in a close proximity to each other and distinctly from the LFD, HFD and HFD-20% WPI group clusters. Indeed, the LFD diet with casein as a protein source clustered most closely with the HFD containing the casein protein.

Phylogenetic analysis revealed several significant microbial population shifts between the HFD control and WPI groups (Table 5). At the family level, all WPI diet groups had significantly increased proportions of *Lactobacillaceae* and

significantly decreased proportions of *Clostridiaceae* compared to the HFD control group. *Bifidobacteriaceae* populations were increased in both the HFD-20% WPI and HFD-30% WPI diet groups compared to the HFD control, while in contrast they were significantly lower in the HFD-40% WPI group compared to the HFD control. The aforementioned patterns were also observed with respect to the corresponding genera (*Lactobacillus*, *Clostridium* and *Bifidobacterium* respectively) (Table 5). Also at genus level, proportions of *Rikenella* were significantly higher in the HFD-40% WPI group compared to HFD-20/30% WPI groups, while proportions of *Peptostreptococcus* were significantly higher in the HFD-40% WPI group than in any other diet group (Table 5). Specific comparison of the microbiota of the HFD-20% WPI and HFD control was deemed particularly important given that the changes occurring here reflected changes resulting specifically from the presence of whey protein, rather than casein, in the diet, and not simply a change in the P/C ratio in the diet. In addition to the changes in the *Lactobacillus*, *Clostridium* and *Bifidobacterium* populations (and associated families) referred to above, it was also noted that proportions of *Desulfovibrio* and *Mucisprillum* (genus) were increased in the HFD-20% WPI, relative to HFD control animals.

## 2.5 DISCUSSION

The key findings of this study are that WPI has a specific effect on HFD-induced energy intake, metabolic health and gut microbiota composition. Additionally, with the exception of energy intake, increasing the P/C ratio, by increasing WPI dietary content, was seen to dramatically alter the above parameters.

Cumulative energy intake measured up to week 16 did not differ between LFD and HFD fed mice. While this is consistent with data reported elsewhere (Pilvi et al. 2007), HF feeding has also been shown to increase or decrease energy intake in rodents (Sclafani et al. 1993, Lin et al. 2000). Differences between data reported may relate to variances in diet composition including fat source/composition, or it may be due to differences in the palatability of the LFD used as the control (Sclafani et al. 1993, West and York 1998).

In this study, there was a discrepancy in energy intake in HFD fed mice depending on the housing environment (single or group housed). In contrast to the group house environment up to week 16, HFD fed mice when individually housed in metabolic cages during weeks 17-20 showed a hypophagic response compared to LFD fed mice. It is possible that these differences may be related to the accuracy of the method used to measure food intake in group versus single housed mice, or alternatively, the different behavioural responses could be result of social isolation, which has been shown to decrease energy intake and elevate plasma corticosterone levels (Saegusa et al. 2011). However, in the latter study this response was found to be transient in nature as socially isolated mice adapted to the new environment and ate similar as grouped housed mice 6 hr post-novelty stress. Indeed, we also showed that group housed mice on a LFD when placed in isolation adapt to the new environment and continue to consume similar food in day 2 and 3 in the new

location [8]. In contrast to mice fed a LFD, mice on a HFD are known to have an over-active hypothalamic-pituitary-adrenal axis (Dallman 2010) resulting in elevated plasma corticosterone levels, as demonstrated here. Consequently, in a HFD background, it's possible that social isolation-induced stress responses could have had a greater impact on energy intake with effects lasting up to or even beyond the 3 day housing period as used in this study. Irrespective of the underlying cause of the hypophagic response in HFD mice, replacing the casein protein with an equivalent WPI content normalised energy intake in HFD-fed mice in a manner similar to that observed in our previous study (McAllan et al. 2013). This appeared to be due to a WPI specific effect on the HFD-induced neuroendocrine state because mice on the LFD with WPI showed similar energy intake to casein diet-fed controls. Since neither WPI nor increasing the P/C ratio influenced plasma corticosterone levels, adipose expression of  $11\beta$ -HSD1 or hypothalamic expression of GCCR in HFD fed mice, it is unlikely that these behavioural changes were due to a WPI effect on stress responses. Given that leptin decreases meal size and number (Hulsey et al. 1998, Bady et al. 2006, Brown et al. 2006), and WPI reduced the HFD-induced increase in plasma leptin levels, it's possible that WPI-derived bioactives could have specifically influenced circadian rhythm of leptin production and/or action within the neuroendocrine state of HFD-fed mice in a socially isolated environment. Additionally, the reduction in plasma amino acids associated with WPI intake (see below), could also have acted as a possible central trigger to increase energy intake in WPI groups compared to HFD control in the single house environment.

HFD feeding has been shown to cause a gain of weight in rats up to the duration of a test period lasting 76 weeks, with animal's body weight gain responding to changes to the dietary fat content introduced at various time points (Peckham et al. 1962).

Lin *et al.*, (Lin *et al.* 2000) demonstrated that mice on a HFD for 19 weeks are responsive to intracerebroventricular administration of leptin. These data suggest that energy balance related mechanisms are able to respond to energetic challenges even after prolonged high fat intake. High protein intake within a HFD suppresses energy intake (Pichon *et al.* 2006, Freudenberg *et al.* 2012) albeit not consistently (Shertzer *et al.* 2011, Schwarz *et al.* 2012), and in our study, the P/C ratio did not alter energy intake in either housing environment. This could be a result of the quantity or composition of the macronutrient used in the test diets. Indeed, data from human trials showed that increasing protein dietary content (10/15% to 30%) only decreased energy intake when the carbohydrate content was kept constant (Weigle *et al.* 2005, Blatt *et al.* 2011). This further highlights the importance of designing appropriate experimental diets with the correct macronutrient composition for uncovering the energy balance related impact of the dietary component under investigation.

Replacement of the casein protein with an equivalent energy content of WPI (i.e. 20%) did not specifically alter metabolic activity, heat production or locomotor activity in HFD or LFD fed mice. In contrast, Acheson *et al.*, (Acheson *et al.* 2011) showed that whey has a greater thermic effect than casein or soy in humans. These differences in data may be related to the fact that the latter study only investigated an acute post-prandial response to a defined test meal, or it may relate to how different species (humans versus mice) digest and metabolise dietary proteins. Shetzer *et al.*, found that mice consuming a HFD and WPI supplemented drinking water have enhanced oxygen consumption compared to mice drinking unsupplemented water (Shetzer *et al.* 2011). In this instance, the increased metabolic activity may have arisen due to the increased protein intake (proteins from diet and from water

supplemented WPI). In fact, this corroborates with the data presented here, which show that increasing the P/C ratio resulted in increased energy expenditure ( $\text{VO}_2$  and heat production) and dark phase locomotor activity, resulting presumably from the increased catabolism of ingested dietary protein, coupled with thermic effects of WPI compared to casein (Acheson et al. 2011) and/or due to increased deposition of lean mass with WPI content (Tipton et al. 2007, Pennings et al. 2011, Kanda et al. 2013). Interestingly, Zhang *et al.* (Zhang et al. 2007) showed that HFD fed mice on leucine supplemented drinking water have reduced fasting plasma levels of aspartic acid, glutamic acid, and phenylalanine, as well as increased  $\text{VO}_2$  and reduced adiposity compared to HFD controls. Given the influence of leucine on WPI-induced muscle hypertrophy (Rieu et al. 2007, Norton et al. 2012) and its unique ability to regulate the translation of protein synthesis (Norton and Layman 2006), it is possible that the elevated leucine content found normally in the WPI diets may have enhanced muscle protein synthesis by directing other amino acids towards protein synthesis and/or catabolism, with the required energy (Garlick et al. 1999, Bender 2012) been derived possibly from fat catabolism (McAllan et al. 2013). Consistent with the latter suggestion, we found an increased lean mass and a reduced fat mass with decreased plasma levels of several amino acids, but not leucine, when the casein protein in a HFD was replaced with WPI or when the WPI P/C ratio in the HFD was increased.

WPI intake appeared to cause a trend towards reduction in fat mass, and in the liver this manifested as a WPI specific reduction in TAG levels, which was accompanied by the suppression of FABP1 mRNA expression, similar to previous findings (Pilvi et al. 2008, Hamad et al. 2011, Shertzer et al. 2011, Shi et al. 2011). In the epididymal adipose tissue, WPI prevented the HFD-induced FASN gene expression,



albeit a recent study reported that WPI does not affect the weight of the epididymal tissue in HFD fed mice, but instead cause a reduction in subcutaneous fat pad weight (Tranberg et al. 2013). These data suggest that WPI affects cellular activity in the liver and in specific adipose tissue depots. While it has been suggested that whey protein may facilitate enhanced postprandial chylomicron clearance via an alteration in LPL expression/activity (Mortensen et al. 2009, Pal et al. 2010), here we did not find a WPI specific effect on LPL expression or plasma TAG levels, but we did observe that intake of the highest P/C ratio (40%-WPI) led to an increase adipose tissue LPL mRNA expression which was accompanied by significant reduction in plasma TAG levels, and a complete reversal of genes involved in lipid accumulation (PPAR $\gamma$ ), fatty acid transport (FATP1), and lipolysis ( $\beta$ 3-AR). Given that HF feeding/obesity down-regulates  $\beta$ 3-AR mRNA expression (Collins et al. 1999), our data suggests an increased adipocyte lipolysis, and reduction in adipose TAG storage in HFD-40%WPI fed mice. Yet the endogenous CPT1b-associated  $\beta$ -oxidation pathway and the UCP-2-associated pathway in epididymal adipose tissue seem to be unaffected (CTP1b) or suppressed (UCP-2) by raising the P/C ratio. This data raises the possibility that the free fatty acids generated from the potentially increased availability of  $\beta$ 3-AR in the adipose may have been re-directed for utilisation by other physiological processes active in HFD-40% group, leading to the increased metabolic activity (VO<sub>2</sub>) observed in the animals. It is also noteworthy that WPI has been shown to increase faecal fat excretion compared to casein (Pilvi et al. 2007), which may have also contributed to the decreased plasma TAG and NEFA seen here with intake of the highest P/C ratio diet (HFD-40% WPI).

Given the link between HFD-induced obesity, low-grade inflammation and insulin resistance (Gustafson et al. 2009, McGillicuddy et al. 2011), one could argue that the

dramatic reduction in fat mass observed with the highest P/C ratio may underlie the effects on inflammatory markers in the adipose tissue (TNF $\alpha$  and CD68) and the hypothalamus (TNF $\alpha$ ), along with simultaneous changes in expression of genes involved in insulin signalling (INSR, IRS-1 and GLUT4 in the adipose, IRS-1 in liver and INSR in the hypothalamus), and the reduction in plasma glucose in these mice. Improvements to insulin sensitivity with WPI have been reported previously (Belobrajdic et al. 2004, Pichon et al. 2008), but our data suggested that only an increased P/C ratio in the HFD facilitated improvements to insulin signalling pathway associated gene expression with prolonged high fat feeding, particularly in the adipose, in parallel with reduced HOMA-IR values.

While many of the effects described above may be due to direct WPI or P/C ratio-host interactions, the effect of WPI and P/C ratio on the composition of the gut microbiota may also play an important role in adiposity and weight gain in these animals. Here, high throughput sequencing based analyses of faecal microbial populations revealed the clustering of the microbiota from animals in receipt of 30 and 40% WPI diets away from those in receipt of 20% kJ WPI or HFD-casein diets. Tranberg *et al* (Tranberg et al. 2013) recently suggested that the efficient absorption of dairy whey proteins in the small intestine may explain the absence of changes in the faecal microbiota. This may explain the clustering of the microbiota from animals fed 20% WPI or HFD-casein diets in our study. However, it is apparent that the high concentrations of WPI present in the 30 and 40% WPI diets employed in our study had a more profound effect, possibly due to additional whey proteins finding their way to the large intestine and/or the overall change in the P/C ratio in the diet. Consumption of the 30 and 40% WPI diets did not result in a shift in the microbiota toward that of the LFD animals and thus the effects on weight gain are

not simply due to an overall conversion to a LFD-like microbiota. Specific taxonomic changes were also noted in response to the different diets. In all cases dietary WPI resulted in significant increases in *Lactobacillaceae/Lactobacillus* and decreases in *Clostridiaceae/Clostridium*. Increased proportions of *Lactobacillus* have previously also been observed in a study of individuals following a regime of calorie restriction and exercise (Santacruz et al. 2009). However, in contrast, increased proportions of *Lactobacillus* have also been noted in HFD fed rats (Parnell and Reimer 2012) and diet-induced obese mice (Clarke et al. 2013). While specific species of *Lactobacillus* have been associated with both lean and obese gut microbiota profiles and also to play a role in obesity and immune response regulation (Ouwehand et al. 1999, Erickson and Hubbard 2000, Armougom et al. 2009), due to the length of the 16S sequences generated and the high degree of sequence homology, we cannot assess changes in proportions of *Lactobacillus* at the species level. An increase in the proportions of *Bifidobacteriaceae/Bifidobacterium* was also observed in both the HFD-20% WPI and HFD-30% WPI compared to the HFD group. This result, combined with the aforementioned increases in *Lactobacillaceae/Lactobacillus*, mirror those reported by Sprong *et al* who suggest that whey proteins act as growth factors for certain species of bacteria by an amino acid composition mediated mechanism (Sprong et al. 2010). Our observations are also consistent with previous findings that high proportions of the class *Clostridiales* are associated with the gut microbiota of animals fed a HFD (Hildebrandt et al. 2009), while fasting reduces the levels of *Clostridium* (Sonoyama et al. 2009). Notably, *Clostridiaceae* can produce short chain fatty acids as a product of their metabolism (Sousa et al. 2007), which can play an important role in the regulation of immune cells and has been associated with inflammation and obesity (Schwartz et al.

al. 2010). These differences, as well as others in the *Proteobacteria Actinobacteria Deferribacteres* (phylum), *Desulfovibrionaceae Deferribacteraceae Veillonellaceae* (family), *Desulfovibrio* and *Mucispirillum* taxa in the HFD-20% whey protein, relative to HFD controls (20% casein) are particularly notable as these reflect changes resulting from the specific presence of whey proteins in the diet, in place of casein, rather than changes in the P/C ratio. Changes in relative proportions may be attributed to (a) the ability of bacteria to utilise whey proteins as a growth medium, (b) the anti-microbial activity of whey protein/peptide components, (c) decreased competition as a result of the whey proteins/peptides antimicrobial activity or (d) even to whey protein mediated changes in the host. Ultimately, the question of cause versus effect remains unanswered, and so while the changes to the microbiota observed may contribute to the mechanisms involved in controlling weight gain, further studies with, for example germ free animals, will be required to determine this definitively.

In summary, our results show that WPI specifically normalises energy intake, increases lean mass and cause a trend towards a reduction in fat mass associated with prolonged high fat feeding. Raising the P/C ratio had no effect on energy intake but augmented metabolic activity and beneficially altered gene expression profiles for lipid metabolism, inflammation and insulin signalling, particularly in the adipose tissue. High throughput analysis of gut microbiota revealed distinct changes in microbial populations with increased P/C ratio causing clustering of 30/40% WPI groups together and distinct from those of HFD and 20% WPI groups, but with specific phylogenetic differences existing between the latter groups. These data indicate that changes to P/C ratio have a dramatic effect on energy balance and the composition of gut microbiota distinct from that seen with changes to protein source.

Future studies should focus on determining whether the effects demonstrated for highest P/C ratio are specific to the WPI content, a consequence of macronutrient change, or both.

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**Table 1.** Plasma amino acid levels ( $\mu\text{mol/L}$ ) in mice fed a 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks

	HFD	20% WPI	30% WPI	40% WPI	P value
Alanine	170.91 $\pm$ 8.82 <sup>a</sup>	163.83 $\pm$ 11.59 <sup>ab</sup>	137.44 $\pm$ 9.72 <sup>ab</sup>	127.15 $\pm$ 8.46 <sup>b</sup>	<.05
Arginine	41.74 $\pm$ 4.82	38.41 $\pm$ 7.16	56.39 $\pm$ 7.60	39.08 $\pm$ 4.34	NS
Aspartic acid	7.47 $\pm$ 1.25 <sup>a</sup>	3.63 $\pm$ 0.51 <sup>b</sup>	3.49 $\pm$ 0.51 <sup>b</sup>	4.85 $\pm$ 1.02 <sup>ab</sup>	<.05
Cyteine	11.95 $\pm$ 1.34	8.87 $\pm$ 1.37	8.93 $\pm$ 1.33	7.86 $\pm$ 1.72	NS
Glutamic acid	90.76 $\pm$ 3.42 <sup>a</sup>	74.74 $\pm$ 1.89 <sup>b</sup>	75.25 $\pm$ 1.86 <sup>b</sup>	71.25 $\pm$ 2.69 <sup>b</sup>	<.001
Glycine	106.22 $\pm$ 4.28 <sup>a</sup>	131.43 $\pm$ 3.57 <sup>b</sup>	116.45 $\pm$ 4.52 <sup>ab</sup>	131.92 $\pm$ 2.69 <sup>b</sup>	<.001
Histidine	75.43 $\pm$ 2.12 <sup>a</sup>	74.93 $\pm$ 2.98 <sup>a</sup>	68.21 $\pm$ 3.35 <sup>ab</sup>	64.21 $\pm$ 2.51 <sup>b</sup>	<.05
Isoleucine	213.81 $\pm$ 8.87	207.87 $\pm$ 9.09	195.27 $\pm$ 5.74	186.78 $\pm$ 10.17	NS
Leucine	91.86 $\pm$ 9.66	81.36 $\pm$ 6.65	66.93 $\pm$ 6.69	69.71 $\pm$ 10.21	NS
Lysine	113.81 $\pm$ 5.88	108.18 $\pm$ 8.30	102.57 $\pm$ 8.39	88.29 $\pm$ 4.07	NS
Methionine	23.94 $\pm$ 1.77	23.65 $\pm$ 0.75	21.05 $\pm$ 0.97	20.40 $\pm$ 1.08	NS
Phenylalanine	46.92 $\pm$ 1.70 <sup>a</sup>	41.13 $\pm$ 1.13 <sup>ab</sup>	38.23 $\pm$ 1.52 <sup>bc</sup>	33.30 $\pm$ 2.35 <sup>c</sup>	<.001
Proline	118.26 $\pm$ 10.14	127.63 $\pm$ 9.63	106.04 $\pm$ 11.02	104.39 $\pm$ 11.93	NS
Serine	68.18 $\pm$ 5.69 <sup>ab</sup>	74.98 $\pm$ 2.29 <sup>a</sup>	62.42 $\pm$ 2.57 <sup>ab</sup>	55.73 $\pm$ 2.12 <sup>b</sup>	<.01
Threonine	92.71 $\pm$ 2.93 <sup>a</sup>	87.63 $\pm$ 2.49 <sup>a</sup>	78.29 $\pm$ 2.95 <sup>ab</sup>	69.28 $\pm$ 3.96 <sup>b</sup>	<.01
Tyrosine	40.29 $\pm$ 2.47 <sup>a</sup>	36.37 $\pm$ 1.12 <sup>ab</sup>	33.76 $\pm$ 1.54 <sup>ab</sup>	29.63 $\pm$ 2.77 <sup>b</sup>	<.01
Valine	154.51 $\pm$ 8.65 <sup>a</sup>	130.59 $\pm$ 5.99 <sup>ab</sup>	118.40 $\pm$ 6.69 <sup>b</sup>	113.16 $\pm$ 9.49 <sup>b</sup>	<.01

Data are means  $\pm$  SEM (n = 7-10). In each row values without a common letter significantly differ, P < 0.05; NS, non-significant

**Table 2.** Tissue lipid parameters and plasma levels of hormones and metabolites in mice fed a 10%kJ low fat diet (LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
Liver TAG (mg/g tissue)	71.47±9.17 <sup>a</sup>	139.03±6.92 <sup>b</sup>	104.94±5.88 <sup>c</sup>	107.21±8.83 <sup>c</sup>	70.82±7.64 <sup>a</sup>	<.001
Leptin (ng/ml)	4.41±0.75 <sup>a</sup>	84.69±3.17 <sup>b</sup>	53.78±5.67 <sup>c</sup>	51.12±4.69 <sup>c</sup>	15.01±4.07 <sup>a</sup>	<.001
TAG (mg/dl)	39.85±2.91 <sup>a</sup>	56.90±6.31 <sup>b</sup>	45.15±4.32 <sup>ab</sup>	52.36±3.91 <sup>ab</sup>	40.19±3.96 <sup>a</sup>	<.05
NEFA (mmol/L)	0.37±0.09 <sup>a</sup>	0.67±0.07 <sup>b</sup>	0.64±0.05 <sup>b</sup>	0.63±0.06 <sup>b</sup>	0.36±0.05 <sup>a</sup>	<.01
Corticosterone (ng/ml)	150.0±21.7 <sup>a</sup>	319.0±48.6 <sup>b</sup>	286.8±31.9 <sup>ab</sup>	334.1±36.7 <sup>b</sup>	277.1±35.5 <sup>ab</sup>	<.01
GLP-1 (pM)	25.03±2.47	33.15±2.83	28.07±2.3	27.88±1.98	24.03±2.25	NS
Glucose (mmol/L)	9.14±1.24 <sup>a</sup>	15.68±0.83 <sup>b</sup>	13.92±0.95 <sup>b</sup>	14.08±1.05 <sup>b</sup>	9.25±0.77 <sup>a</sup>	<.001
Insulin (ng/ml)	0.29±0.05	0.55±0.08	0.40±0.10	0.37±0.04	0.31±0.06	NS
HOMA-IR	2.48±0.67 <sup>a</sup>	9.79±1.07 <sup>b</sup>	6.81±2.58 <sup>ab</sup>	6.20±0.95 <sup>ab</sup>	3.04±0.64 <sup>a</sup>	<.01

Data are means ± SEM (n = 5-10). In each row results without a common letter significantly differ,  $P \leq 0.05$  NS, non-significant.

TAG, triacylglycerol. NEFA, non-esterified fatty acids. GLP-1, glucagon-like peptide

1. HOMA-IR, homeostasis model assessment of insulin resistance.

**Table 3.** Relative hepatic gene expression in mice fed a 10%kJ low fat diet (LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
CD36	1.00±0.20 <sup>a</sup>	2.85±0.34 <sup>b</sup>	2.98±0.45 <sup>b</sup>	2.17±0.25 <sup>ab</sup>	1.50±0.25 <sup>a</sup>	<.05
PPAR $\gamma$	1.00±0.12 <sup>a</sup>	3.00±0.57 <sup>b</sup>	2.30±0.49 <sup>b</sup>	1.88±0.46 <sup>b</sup>	0.85±0.13 <sup>a</sup>	<.001
FABP1	1.00±0.09 <sup>ab</sup>	1.27±0.10 <sup>b</sup>	0.86±0.06 <sup>ac</sup>	1.27±0.17 <sup>bc</sup>	0.71±0.09 <sup>a</sup>	<.001
IRS-1	1.00±0.15 <sup>a</sup>	0.58±0.04 <sup>b</sup>	0.69±0.05 <sup>ab</sup>	0.68±0.06 <sup>ab</sup>	0.79±0.06 <sup>ab</sup>	<.01
CPT1a	1.00±0.08	0.85±0.08	0.97±0.06	0.90±0.13	0.86±0.10	NS
GLUT2	1.00±0.09	0.76±0.04	1.02±0.08	0.97±0.09	0.89±0.05	NS
FATP5	1.00±0.21	0.89±0.07	0.88±0.07	1.16±0.07	1.24±0.12	NS
FASN	1.00±0.36	0.46±0.14	0.54±0.19	0.40±0.06	0.32±0.04	NS
PPAR $\alpha$	1.00±0.04	1.13±0.05	1.21±0.05	1.17±0.10	1.28±0.11	NS
TNF $\alpha$	1.00±0.26	0.84±0.15	1.12±0.18	0.80±0.09	0.71±0.10	NS

Data are means  $\pm$  SEM (n = 7-10). In each row results without a common letter significantly differ,  $P < 0.05$ ; NS, non-significant.

Gene expression shown relative to the LFD control group set at 1.00.

CD36, cluster of differentiation 36; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma; FABP1, Fatty acid binding protein 1; IRS-1, Insulin receptor substrate 1; CPT1a, carnitine palmitoyltransferase 1a; GLUT2, Glucose transporter 2; FATP5, Fatty acid transporter 5; FASN, Fatty acid synthase; TNF- $\alpha$ , Tumour necrosis factor alpha.

**Table 4.** Relative hypothalamic gene expression in mice fed a 10%kJ low fat diet

(LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
IR	1.00±0.03 <sup>ab</sup>	0.97±0.07 <sup>a</sup>	1.22±0.04 <sup>bc</sup>	1.37±0.04 <sup>cd</sup>	1.55±0.08 <sup>d</sup>	<.05
GCCR	1.00±0.09 <sup>a</sup>	1.40±0.10 <sup>b</sup>	1.34±0.07 <sup>ab</sup>	1.30±0.08 <sup>ab</sup>	1.42±0.09 <sup>b</sup>	<.05
TNF $\alpha$	1.00±0.05 <sup>a</sup>	1.76±0.09 <sup>b</sup>	1.57±0.13 <sup>b</sup>	1.47±0.14 <sup>ab</sup>	1.40±0.25 <sup>ab</sup>	<.01
POMC	1.00±0.08	0.99±0.06	1.01±0.06	0.98±0.06	1.00±0.08	NS
NPY	1.00±0.04	1.02±0.09	0.90±0.06	0.88±0.05	0.92±0.08	NS
ObR	1.00±0.11	0.92±0.05	0.99±0.05	1.07±0.07	0.95±0.05	NS
GHS-R	1.00±0.07	0.98±0.06	0.98±0.08	1.02±0.11	1.04±0.09	NS
PPAR $\gamma$	1.00±0.36	1.07±0.04	0.95±0.05	0.99±0.05	0.98±0.07	NS
CPT1c	1.00±0.09	0.92±0.04	0.91±0.01	0.97±0.06	0.86±0.04	NS
IRS-1	1.00±0.04	0.99±0.04	0.97±0.02	1.06±0.08	1.00±0.06	NS

Data are means  $\pm$  SEM (n = 5-10). In each row values without a common letter significantly differ,  $P < 0.05$ ; NS, non-significant.

Gene expression shown relative to the LFD control group set at 1.00.

IR, Insulin receptor; GCCR, Glucorticoid receptor; TNF- $\alpha$ , Tumour necrosis factor alpha; POMC, Pro-opiomelancortin; NPY, Neuropeptide Y; ObR, Leptin receptor; GHS-R, Growth hormone secretatogue receptor; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma; CPT1c, carnitine palmitoyltransferase 1c IRS-1, Insulin receptor substrate 1.

**Table 5.** Gut microbiota composition as % of reads in mice fed a 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks

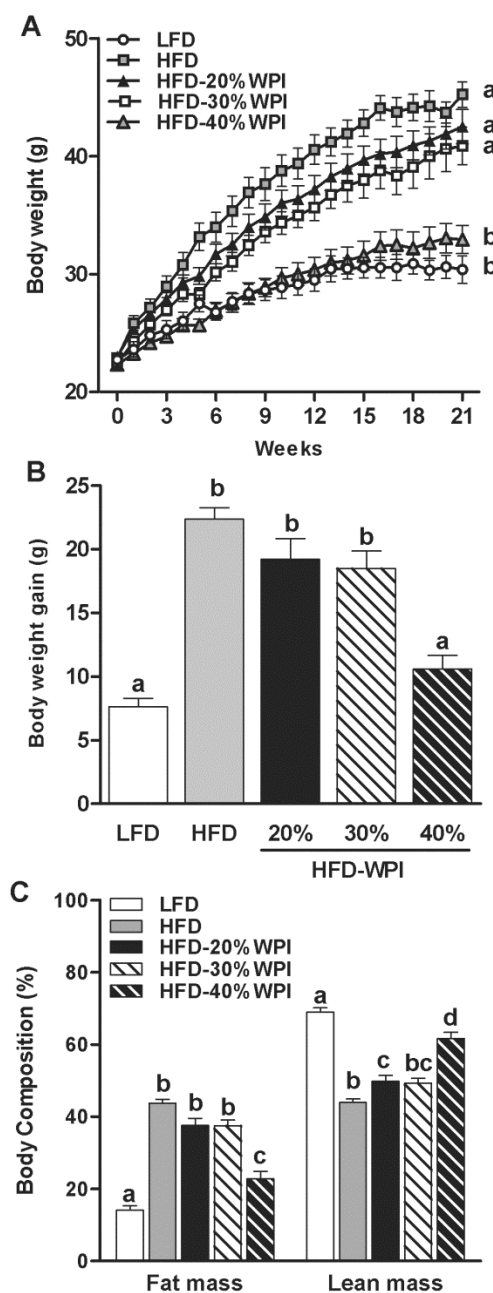
	HFD	20% WPI	30% WPI	40% WPI
<b>Phylum</b>				
<i>Proteobacteria</i>	0.36 <sup>a</sup>	0.63 <sup>b</sup>	0.34 <sup>ab</sup>	0.32 <sup>a</sup>
<i>Actinobacteria</i>	0.63 <sup>a</sup>	1.82 <sup>b</sup>	3.79 <sup>b</sup>	0.36 <sup>c</sup>
<i>Deferribacteres</i>	0.57 <sup>a</sup>	1.61 <sup>b</sup>	1.56 <sup>ab</sup>	2.03 <sup>b</sup>
<b>Family</b>				
<i>Desulfovibrionaceae</i>	0.12 <sup>a</sup>	0.31 <sup>b</sup>	0.21 <sup>ab</sup>	0.23 <sup>ab</sup>
<i>Rikenellaceae</i>	6.71 <sup>ab</sup>	7.54 <sup>b</sup>	3.9 <sup>a</sup>	6.4 <sup>ab</sup>
<i>Bacteroidaceae</i>	0.44 <sup>a</sup>	0.42 <sup>a</sup>	0.16 <sup>b</sup>	0.21 <sup>b</sup>
<i>Lactobacillaceae</i>	0.21 <sup>a</sup>	3.03 <sup>b</sup>	4.6 <sup>b</sup>	2.14 <sup>b</sup>
<i>Bifidobacteriaceae</i>	0.43 <sup>a</sup>	1.71 <sup>b</sup>	3.66 <sup>b</sup>	0.22 <sup>c</sup>
<i>Deferribacteraceae</i>	0.57 <sup>a</sup>	1.59 <sup>b</sup>	1.32 <sup>ab</sup>	2.03 <sup>ab</sup>
<i>Peptostreptococcaceae</i>	0.62 <sup>a</sup>	1.79 <sup>a</sup>	1.54 <sup>a</sup>	8.01 <sup>b</sup>
<i>Succinivibrionaceae</i>	0.13 <sup>a</sup>	0.15 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>
<i>Clostridiaceae</i>	1.31 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>
<i>Veillonellaceae</i>	0.02 <sup>a</sup>	0.12 <sup>b</sup>	0 <sup>a</sup>	0 <sup>a</sup>
<b>Genus</b>				
<i>Anaerobiospirillum</i>	0.13 <sup>a</sup>	0.15 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>
<i>Desulfovibrio</i>	0.07 <sup>a</sup>	0.22 <sup>b</sup>	0.17 <sup>ab</sup>	0.15 <sup>ab</sup>
<i>Alistipes</i>	4.33 <sup>ab</sup>	4.41 <sup>a</sup>	2.24 <sup>b</sup>	3.76 <sup>ab</sup>
<i>Rikenella</i>	1.04 <sup>ab</sup>	0.49 <sup>b</sup>	0.68 <sup>b</sup>	1.08 <sup>b</sup>
<i>Bacteroides</i>	0.44 <sup>a</sup>	0.4 <sup>a</sup>	0.16 <sup>b</sup>	0.21 <sup>ab</sup>
<i>Oscillibacter</i>	0.24 <sup>a</sup>	0.67 <sup>ab</sup>	0.42 <sup>ab</sup>	0.52 <sup>b</sup>
<i>Lactobacillus</i>	0.2 <sup>a</sup>	3.03 <sup>b</sup>	4.6 <sup>b</sup>	2.39 <sup>b</sup>
<i>Bifidobacterium</i>	0.43 <sup>a</sup>	1.71 <sup>b</sup>	3.66 <sup>b</sup>	0.22 <sup>c</sup>
<i>Mucispirillum</i>	0.57 <sup>a</sup>	1.61 <sup>b</sup>	1.56 <sup>ab</sup>	1.92 <sup>ab</sup>
<i>Coproccoccus</i>	0.11 <sup>ab</sup>	0.23 <sup>b</sup>	0.06 <sup>a</sup>	0.06 <sup>ab</sup>
<i>Turicibacter</i>	0.56 <sup>a</sup>	0.35 <sup>a</sup>	0.15 <sup>ab</sup>	0 <sup>b</sup>
<i>Clostridium</i>	1.3 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>
<i>Peptostreptococcus</i>	0.1 <sup>a</sup>	0.14 <sup>a</sup>	0.12 <sup>a</sup>	0.78 <sup>b</sup>

Data are means  $\pm$  SEM (n = 10). Statistically significant differences generated using the

Kruskal-Wallis algorithm. In each row values without a common letter significantly differ, *P*

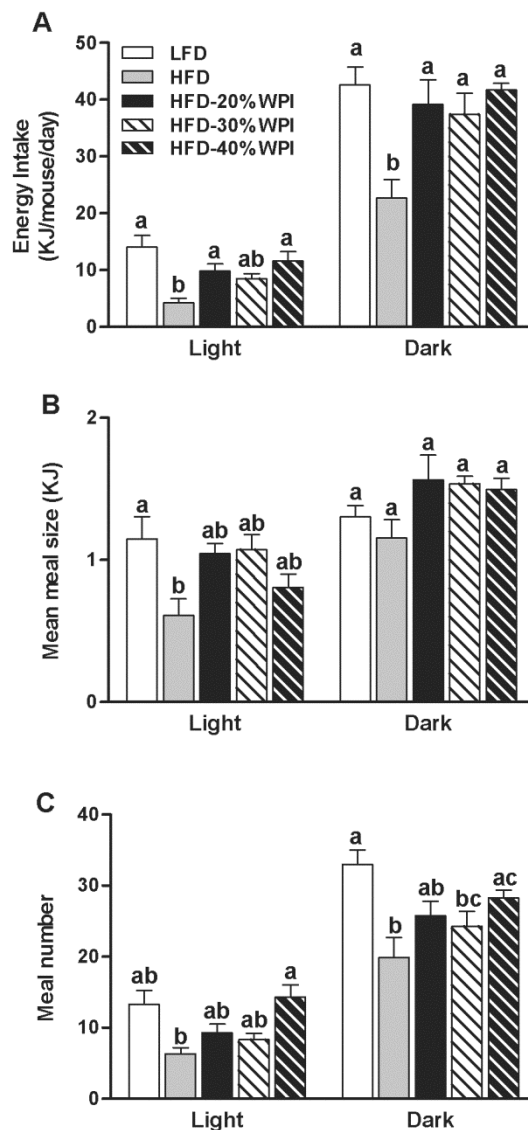
$\leq 0.05$ .

**Figure. 1. Impact of whey protein isolate and protein to carbohydrate ratio on body weight and composition.** (A) shows the body weight trajectories of mice during 21 weeks of dietary treatment with a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Body weight gain (B) and body composition (C) of mice after 21 weeks on experimental diets are also shown. Data represent mean values  $\pm$  S.E.M. (n = 10 per group). Groups that do not share a common letter are significantly different at  $P < 0.05$ .

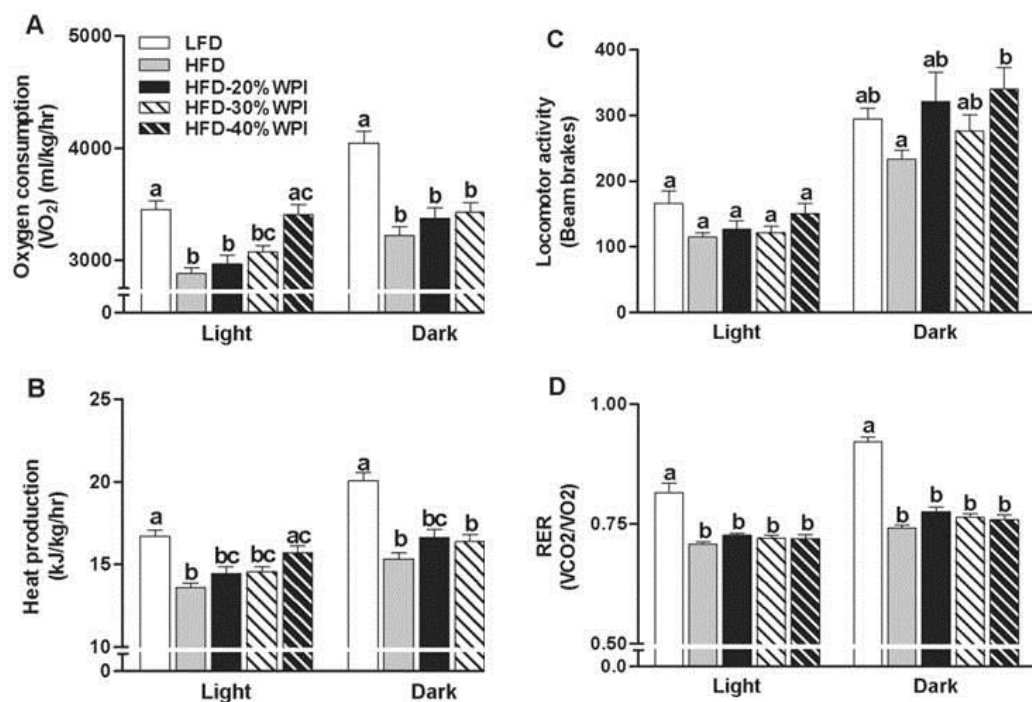




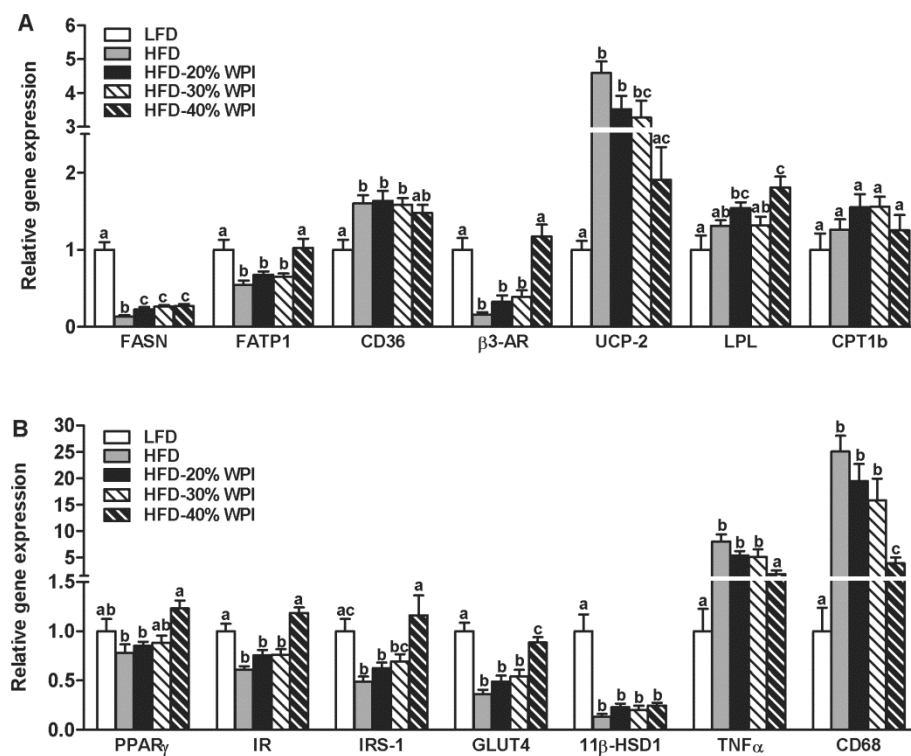
**Figure. 2. Impact of whey protein isolate and protein to carbohydrate ratio on energy intake.** Energy intake (A) and feeding behaviour (meal size & meal number) (B-C) was measured using TSE Phenomaster cages at 17-20 weeks for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data collected from individual mice at 9 minute intervals over a 24 hour period are shown as mean values  $\pm$  SEM (n = 8-10 per group) for light and dark phases. In light and dark phase, groups that do not share a common letter are significantly different at  $P < 0.05$ .



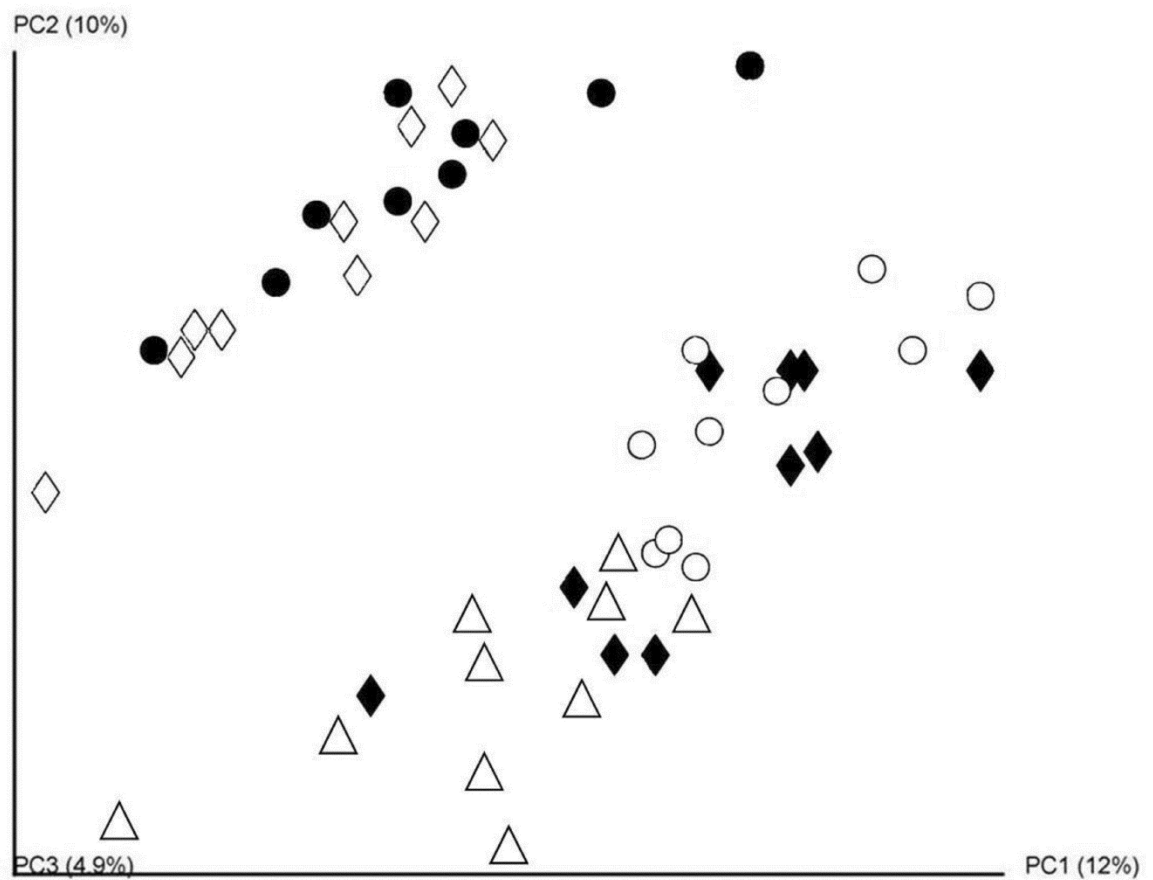
**Figure 3. Impact of whey protein isolate and protein to carbohydrate ratio on metabolic activity.** Metabolic activity was measured using TSE Phenomaster cages at 17-20 weeks for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data for (A) oxygen consumption ( $\text{VO}_2$ ), (B) heat production, (C) locomotor activity and (D) respiratory exchange ratio (RER), collected from individual mice at 9 minute intervals over a 24 hour period, are shown as mean values  $\pm$  SEM (n = 8-10 per group) for light and dark phases. In light and dark phase, groups that do not share a common letter are significantly different at  $P < 0.05$ .



**Figure 4. Impact of whey protein isolate and protein to carbohydrate ratio on adipose cellular activity.** Epididymal adipose tissue gene expression was investigated in mice after 21 weeks on a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD), or HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Relative mRNA expression is shown for (A) fatty acid synthase (FASN), fatty acid transporter 1 (FATP1), cluster of differentiation 36 (CD36), beta-3 adrenergic receptor ( $\beta$ 3-AR), uncoupling protein 2 (UCP-2), lipoprotein lipase (LPL) and carnitine palmitolytransferase 1b (CPT1b), and (B) for peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), insulin receptor (IR), insulin receptor substrate 1 (IRS-1), glucose transporter 4 (GLUT4), 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1), tumour necrosis factor alpha (TNF- $\alpha$ ) and cluster of differentiation 68 (CD68). Data represent mean values  $\pm$  SEM (n = 9-10 per group). Gene expression is shown relative to the LFD control group set at 1.00. Groups that do not share a common letter are significantly different at  $P < 0.05$ .



**Figure 5. Impact of whey protein isolate and protein to carbohydrate ratio on the gut microbiota composition.** Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of the 16SrRNA sequences, demonstrating where sequences cluster according to diet group. Data were generated from analysis of faecal samples collected from mice on 10% kJ low fat diet (LFD,  $\Delta$ ) or 45% kJ high fat diet (HFD,  $\blacklozenge$ ) or a HFD with 20% kJ whey protein isolate (HFD-20% WPI,  $\circ$ ), 30% kJ WPI (HFD-30% WPI,  $\diamond$ ) or 40% kJ WPI (HFD-40% WPI,  $\bullet$ )(n=10).



## **Chapter 3**

### **Impact of the dairy whey proteins lactoferrin and serum albumin on the composition of the gut microbiota**

#### **Peter Skuse Chapter Contributions**

##### **Experimental:**

- Designed and performed all experiments relating to *ex vivo* distal colon model and all fecal sample DNA extraction, PCR amplification DNA sequencing from *in vivo* experiments.

##### **Results interpretation:**

- Analysis of all data pertaining to 16S rRNA gene sequencing.

##### **Manuscript preparation:**

- Major contributor to manuscript preparation.

### 3.1 ABSTRACT

The obesity pandemic has become perhaps the most prevalent health issue of our time. Although, obesity is primarily caused by a long term positive energy imbalance, it is becoming increasingly appreciated that the composition and functionality of our enteric microbial communities can greatly influence the pathogenesis of obesity and related conditions. By extension, it is a reasonable assumption that selective modulation of the gut microbiota may yield novel treatments for diseases such as obesity. Whey proteins have been shown to reduce body weight and to improve the outcomes in obesity related conditions such as type-2-diabetes. However, their effect on the gut microbiota is poorly understood. We previously demonstrated, in C57BL/6J mice, that the replacement of casein as the dietary protein source with whey protein isolate (WPI) reduced the animal's body weight and enriched enteric populations of *Lactobacillus* and *Bifidobacterium*. However the large array of bioactive proteins and associated peptides contained within WPI makes drawing conclusions between specific protein sources and alterations to the gut microbiota difficult. Therefore this study employed a two armed approach and next generation high-throughput DNA sequencing technologies to evaluate the impact of two component proteins of whey, namely bovine serum albumin (BSA) and lactoferrin (Lf), on the composition and diversity of 1) the human gut microbiota by means of an *ex vivo* distal colon anaerobic fermentation system and 2) the gut microbiota of C57BL/6J mice in receipt of a high fat diet (HFD). Results from the simulated colonic fermentation revealed that BSA did not invoke significant direct alterations of the gut microbiota. Furthermore, native Lf (nLf), Lf which had undergone pre-processing by heat treatment (hLf) or

conjugation with fructooligosaccharide (FOS) (mLF) followed by an upper GI simulated gastric digestion, all invoked similar and subtle alterations to the human gut microbiota, including the enrichment of bacteria such as *Veillonellaceae*, *Acidominococcus* and *Phascolarctobacterium*. In mice, replacement of casein as the protein source of a high fat diet with either BSA or Lf reversed several of the high fat associated taxonomic shifts and, in the case of Lf, significantly increased the proportions of the *Roseburia* genus. PICRUST analysis revealed that the microbiome of both BSA and Lf-fed animals was more akin to that of LFD fed animals. These results suggest a use for bovine whey proteins in the beneficial modulation of the gut microbiota.

### 3.2 INTRODUCTION

The WHO estimated that, in 2014, 1.9 billion people worldwide were overweight, of which nearly 600 million were obese. Obesity is a complex multifactorial condition which primarily occurs as a result of a long term positive energy balance. The increase in the incidence of obesity over the last 20-25 years to its current epidemic status has been associated with modern eating habits combined with an increased sedentary lifestyle. However, other factors, such as host genetics and the indigenous microbial populations, have also been shown to contribute to the onset of obesity (Ley et al. 2006, Thorleifsson et al. 2009). While the rapid rise of obesity has been well documented, there has been a failure in the treatment of the current epidemic with either sustained lifestyle changes or medical interventions (Wing and Hill 2001, Fallah-Fini et al. 2014, Roberto et al. 2015). Obese individuals are at a considerably higher risk of developing several clinical conditions such as type 2 diabetes, hypertension and cardiovascular disease (Eckel et al. 2005). Given these associated increased health risks coupled with the considerable economic cost of treating obesity and obesity related diseases (Finkelstein et al. 2009, Lobstein 2014), there is an urgent requirement to develop novel strategies for the long term treatment and prevention of obesity.

One such proposed novel strategy involves the beneficial modulation of gut microbial populations (Walsh et al. 2014). The gut microbiota is predominantly comprised of bacterial species which are members of the Firmicutes and Bacteroidetes phyla, with the remaining species being members of the Actinobacteria, Verrucomicrobia, Proteobacteria, Fusobacteria and Cyanobacteria phyla (Qin et al. 2010). It is estimated that over 1000 bacterial species are contained within the human microbiota, each of which possesses a genome encoding an



average of ~5,000 genes (Consortium 2012). The human microbiota and associated metagenome therefore carries with it a genetic repertoire which far outweighs that of the human host (~23,000 genes) and is capable of carrying out metabolic functions not encoded by the human genome. The vast majority of these microbial inhabitants (1.5 kg or 100 trillion cells) reside within the gut (Lander et al. 2001, Eckburg et al. 2005, Consortium 2012). The gut microbiota has been shown to impact on obesity through several different mechanisms including the production of short chain fatty acids (SCFAs), modulation of host inflammation and increased dietary calorie extraction (Bäckhed et al. 2004). Initially, an increase in the Firmicutes : Bacteroidetes (*F:B*) ratio was noted in studies with obese rodents (Ley et al. 2005) and humans (Ley et al. 2006) leading to an increased *F:B* ratio being proposed as a potential biomarker of obesity. However these findings have been the subject of much debate (Murphy et al. 2010, Schwartz et al. 2010) and, indeed, other studies attributed this shift in the dominant gut phyla to high fat feeding which was independent of the obese state (Hildebrandt et al. 2009). Regardless of this issue, several studies, particularly those using germ free (GF) mice, have highlighted the importance of the gut microbiota in the development of obesity. Initially, it was shown that GF mice are protected from diet induced obesity while their conventionalised counterparts are not, thus highlighting the importance of the gut microbiota in the aetiology of obesity (Bäckhed et al. 2007, Ridaura et al. 2013). Furthermore, other GF studies have shown that the obese-associated microbiota may be more efficient at harvesting dietary calories in that GF mice conventionalised with a microbiota from genetically obese (*ob/ob*) mice had an increase of body fat when compared to mice conventionalised with a microbiota from lean animals (Turnbaugh et al. 2006). While the exact components and activities of the gut

microbiota that influence obesity require further investigation, it is clear that gut microbial composition is altered in obesity as well as obesity related conditions such as metabolic syndrome and type 2 diabetes, and thus modulation of these obesity related shifts in microbial populations and diversity might in turn aid in the treatment and prevention of obesity.

Dairy proteins originating from the whey fraction (20% of total milk proteins) have been shown to possess anti-obesity properties. More specifically, incorporation of dietary whey protein has been shown to reduce body weight and fat mass accumulation in both high fat diet (HFD) fed rodents (Shertzer et al. 2011, Shi et al. 2011) and obese humans (Pal et al. 2010, Baer et al. 2011). Whey contains several bioactive proteins such as  $\alpha$ -lactoglobulin,  $\beta$ -lactalbumin, lactoferrin and serum albumin, some of which have been shown to possess antimicrobial activity (Yamauchi et al. 1993, Shin et al. 2000). Furthermore, digestion of these proteins may result in the formation of other antimicrobial peptides, such as the pepsin catalysed formation of the antimicrobial peptide lactoferricin (Lfcin) from lactoferrin (Hoek et al. 1997). While studies have examined the effect of whey proteins in terms of body weight, to date few studies have examined the effect of these milk derived proteins on enteric microbial populations. Two such studies, by McAllan *et al.* and Sprong *et al.*, reported an increase in the proportions of both the *Lactobacillus* and *Bifidobacterium* genera when the dietary protein energy source of casein was substituted with whey. However, these results were not replicated in other such studies (Sprong et al. 2010, Tranberg et al. 2013, McAllan et al. 2014). The use of whey protein isolate (WPI) makes drawing conclusions between the specific proteins contained within whey (and associated digested peptides), and the effect which they may invoke on the gut microbial populations, difficult. Therefore

this study aimed to examine the effect of two whey proteins, namely bovine serum albumin (BSA) and lactoferrin (Lf), on the adult human microbiota in an *ex vivo* distal colon model and the microbiota of C57BL/6 mice consuming a high fat diet (HFD). To our knowledge, it is the first study to examine the effect of specific whey proteins on enteric microbial populations.

### **3.3 MATERIALS AND METHODS**

#### **3.3.1 Materials used in distal colon model experiments**

BSA was purchased from Sigma Aldrich, USA. Food grade bovine Lf (Vivinal lactoferrin TD, 95.6% protein) was kindly donated by FrieslandCampina (Wageningen, Netherlands) and oligofructose (Orafti®P95, 95% oligofructose) (FOS) was kindly donated by BENEIO (Universal Network LTD, Rosh HaAyin, Israel). Fresh (frozen) porcine bile used for *in vitro* gastro-intestinal digestion was acquired from Lahav Research Institute (Kibbutz Lahav, Israel). All other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared with deionized water (DW) and all reagents were of analytical grade.

#### **3.3.2 Preparation of Maillard reaction products (MRPs) and heated Lf**

Lf-based MRPs were prepared by mixing native lactoferrin with FOS as detailed in previous work (Moscovici et al. 2014). Briefly, the protein powder was dissolved with FOS at a practical 3:1 w/w ratio in DW while mixing at ambient temperature for 4 h. The solution was adjusted to pH 7.0 with 1 M NaOH, freeze dried and pulverized into a fine powder which was incubated at 60 °C under water restricted conditions (79 % RH achieved over saturated potassium bromide) for 24 h. Heated Lf was produced similarly without the addition of FOS, to isolate the thermal effect from the Maillard reaction. Finally, resulting samples were dialyzed to remove unreacted carbohydrates (dialysis tubing 12-14 kDa MW cut-off, Thomas Scientific, Swedesboro, NJ). Dialyzed samples were then lyophilized, pulverized and kept in a desiccator until further analysis. Lf-FOS MRPs were termed mLf, heated Lf was termed hLf. The native protein control (not heated) was termed nLf.

### 3.3.3 *In vitro* adult gastro-intestinal digestion

*In vitro* adult gastro-intestinal digestion of nLf, hLf and mLf was performed according to a recently standardized static method by COST action FA1005 INFOGEST (Minekus et al. 2014) with slight modifications. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were also prepared according to Minekus *et al.* Briefly, five parts of a 0.05 g/ml sample solution dissolved in distilled water (DW) was mixed with four parts of SGF. After mixing and incubation at 37 °C for 10 min, DW, CaCl<sub>2</sub>, 1 M HCl and porcine pepsin were added to achieve 2000 U/ml pepsin, 0.075 mM CaCl<sub>2</sub> and pH 3.0 in the final reaction mixture. Digesta aliquots were withdrawn before pepsin addition and at different time points along the gastric phase: after 5, 60 and 120 min. These were rapidly neutralized with 0.5 M ammonium bicarbonate and placed on ice, and were denoted: G0 for a sample before pepsin addition, G5 for a sample withdrawn 5 min after pepsin addition and so-forth.

After 2h of gastric phase, five parts of gastric chyme were mixed with four parts of SIF followed by the addition of 1 M NaOH, DW, CaCl<sub>2</sub>, porcine bile, trypsin and  $\alpha$ -chymotrypsin to achieve 0.3 mM CaCl<sub>2</sub>, 10 mM bile, 100 U/ml trypsin, 25 U/ml  $\alpha$ -chymotrypsin and pH 7.0 in the final reaction mixture. To partly mimic absorption of low MW digestion products similarly to previous reports (Mills et al. 2008), the reaction mixture was dialyzed against SIF while mixing at 37 °C for 2 h (dialysis tubing 1 kDa MW cut-off, Membrane Filtration Products Inc., Seguin, TX). Digesta aliquots were taken 60 and 120 min after addition of enzymes, and indexed I60 and I120, respectively. These aliquots were rapidly mixed with the serine protease inhibitor phenylmethanesulfonylfluoride (PMSF) and placed on ice.

The remaining digesta following gastro-intestinal digestion was snap-freezed in liquid nitrogen and freeze dried to be used as samples for colonic fermentations as detailed below.

### **3.3.4 Monitoring gastro-intestinal proteolysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Comparison of peptide breakdown profiles of digesta samples was based on SDS-PAGE as described in previous works (Moscovici et al. 2014).

### **3.3.5 Distal Colon Model**

Fecal media, prepared according to Fooks *et al* (Fooks and Gibson 2003), was autoclaved along with the vessels of the Multifors fermentation system (Infors, UK) at 121°C for 15 minutes. Once cooled, the media was adjusted to pH 6.8 and added (160 ml) to each of the fermentation vessels. Proteins of interest were then added to the media (as appropriate, see below) and vessels were sparged with N<sub>2</sub> for 120 minutes to establish anaerobic conditions. A human microbiota was inoculated into the vessels of the fermentation system by means of a fresh composite fecal slurry. On the morning of each experiment, fresh fecal samples were collected from three healthy donors, aged 20-40 years, who had not used antibiotics within the previous six months. A 20% (BSA experiment) or 10% (Lf experiment) fecal slurry was made by homogenising weighed fecal samples with previously boiled and cooled phosphate buffer solution containing 0.05% cysteine in a sterile stomacher bag for one minute. Immediately after homogenising, 40ml of the fecal slurry was inoculated into each vessel to achieve a total volume of 200 ml. Throughout the experiment, pH was maintained at 6.8 by the automatic addition of 1M NaOH or HCL (as appropriate), incubated at 37°C, continuously mixed with an automatic

stirrer (100 rpm) and constantly sparged with N<sub>2</sub> to maintain anaerobic conditions. Experiments were carried out in triplicate using the same fecal donors for each run. For the BSA experiment, BSA () was added to two of the three fermentation vessels to achieve final concentrations of 2.5% (5g BSA) and 5% (10g BSA), respectively. No BSA was added to the third vessel, which served as a control. For Lf experiments, 2g of nLf, hLf and mLf were added to their respective fermentation vessels to give a total final concentration of 1%. 2g of FOS was added to another fermenter to serve as a positive control. Three mls of fermentate were drawn out of each vessel at each sampling point. Drawn off samples were then centrifuged at 15,000 x rpm for 15 minutes where, the remaining pellet was used for DNA extraction and subsequent compositional sequencing as detailed below. For the BSA experiment, samples were drawn from each vessel at 0h and 24h after fecal slurry inoculation. For the Lf experiment, samples were drawn from each vessel at 0, 4, 8 and 24h after fecal slurry inoculation.

### **3.3.6 Animals and diets**

The use and care of animals adhered to the license obtained under the Cruelty to Animals Act 1876. All subsequent work was in accordance with University College Cork Animal Ethics Committee approval (#2011/005). Weight matched ( $17.89 \pm 1.68$ g) male C57/BL6J mice (Harlan, Middlesex, UK) aged 3-4 weeks were randomly group housed in individually ventilated cages (4 per cage, 8 per group) in a light (06:00-18:00), temperature ( $21 \pm 1^{\circ}\text{C}$ ) and humidity (45-65%) controlled environment. Mice were given *ad libitum* access to fresh water and one of three diets (Research Diets): LFD (10% energy fat and 20% energy casein; D12450B), HFD (45% energy fat and 20% energy casein; D12451), HF-BSA (D12451 in which BSA

replaced 20% energy casein, BSA; Sigma Aldrich, USA) or HF-Lf (D12451 in which Lf replaced 20% energy casein, Lf; Glanbia, USA).

### **3.3.7 *In vivo* experimental protocol**

Experimental protocol was carried out as per McManus *et al.* (McManus et al. 2015, McManus et al. 2015) After 12 weeks of the trial, fecal pellets were taken from individual mice for DNA extraction and subsequent compositional sequencing.

### **3.3.8 DNA extraction**

DNA was extracted from centrifuged fermentate pellets and individual murine fecal pellets using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK), which was combined with an additional bead beating step (30s x 3). Extracted DNA was quantified using the Nanodrop 1000 spectrophotometer and stored at -20°C. Sequencing was performed on the Roche 454 GLS FLX genome sequencing platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) or Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA).

### **3.3.9 Roche 454 high-throughput pyrosequencing**

Bacterial composition was determined by high-throughput DNA sequencing of 16s rRNA amplicons (V4-V5 region; 408bp long) generated by a separate PCR reaction (in triplicate) for each sample. Universal 16s rRNA primers designed to bind to an estimated 94.6% of all 16s genes present were employed for PCR amplification where forward primer (5'-AYTGGGYDTAAAGNG), with attached molecular identifier tags between the 454 adapter sequence and target-specific primer sequence, and reverse primer V5 (5'-CCGTCAATTYYTTTTRAGTTT) were used (Claesson et al. 2010) in combination with Biomix Red (Bioline, London, UK.) PCR



amplification of template DNA was carried out under the following conditions for a total of 35 cycles: 94°C for 2 minutes and 1 minute respectively (initialization and denaturation), 56°C for 1 minute (annealing) and 72°C for 1 minute (elongation) followed by a final elongation step of 2 minutes. A negative control, whereby template DNA was substituted with PCR grade water, was employed and did not yield an amplicon. Amplicons were subsequently cleaned using the Ampure purification system (Beckman and Coulter, Takeley, UK), quantified using the Quant-it™ Picogreen® ds DNA Assay Kit (Invitrogen, Waltham, MA, USA) coupled with the NANODROP 3300 Fluorospectrometer (Thermo Scientific, USA) and finally pooled to an equimolar concentration. Samples were then sequenced in house on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in accordance to manufactures protocols.

### **3.3.10 Illumina MiSeq 16s rRNA amplicon sequencing**

The V3-V4 variable region of the 16s rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina, CA, USA). Initially the template DNA was amplified using primers specific to the V3-V4 region of the 16s rRNA gene which also allowed for the Illumina overhang adaptor, where, the forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse primer (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used. Each PCR reaction contained 2.5 µl DNA template, 5 µl forward primer (1µM), 5 µl reverse primer (1µM) (Sigma, Ireland) and 12.5 µl Kapa HiFi Hotstart Readmix (2X) (KAPA Biosystems Inc, USA). The template DNA was amplified under the following PCR conditions for a

total of 25 cycles: 95°C for 3 minutes and 30 seconds respectively (initialization and denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (elongation) followed by a final elongation period of 5 minutes. A negative control reaction whereby the template DNA was replaced with PCR grade water was employed to confirm lack of contamination and PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose gel, 100v) post PCR reaction. Successful amplicons were then cleaned using the AMPure XP purification system (Labplan, Dublin, Ireland). A second PCR reaction was then completed using the previously amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, CA, USA) were used per sample to allow all samples to be pooled, sequenced on one flow cell and subsequently identified bioinformatically. Each reaction contained 25 µl Kapa HiFi HotStart ReadyMix (2X), 5 µl template DNA, 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx) and 10 µl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just 8 cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 fluorometer (Invitrogen) in conjunction with the broad range DNA quantification assay kit (Life technologies, Carlsbad, CA, USA). All samples were then pooled to an equimolar concentration and the pool underwent a final cleaning step. Quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2M NaOH, diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX. Samples were then sequenced in house on the MiSeq sequencing platform using a 2.300 cycle V3 Kit following protocols outlined by Illumina.

### **3.3.11 Bioinformatic analysis**

Raw Roche 454 high-throughput sequence data was quality trimmed using a locally installed RDP pyrosequencing pipeline where, reads shorter than 150bp, ambiguous bases and non-exact primer matches were removed. The trimmed FASTA files were then BLASTed (Altschul et al. 1997) against a locally installed version of SILVA 16s rRNA database (Pruesse et al. 2007) using default parameters. Resulting BLAST output files were then parsed through MEGAN (Huson et al. 2007) which assigns National Centre for Biotechnology Information (NCBI) taxonomies using the lowest common ancestor algorithm. Bit scores were employed to allow for the filtering of results prior to tree construction and summarization where, a bit score of 86 was employed from within MEGAN (Urich et al. 2008, Rea et al. 2011). Sequence reads were clustered into operational taxonomic units (OTUs) using the QIIME software suite (Caporaso et al. 2010). Chimeric OTUs were removed using the Chimeraslayer program and phylogenetic trees constructed using the FastTreeMP tool (Price et al. 2010, Haas et al. 2011). Beta diversity values were calculated based on Bray Curtis, weighted and unweighted Unifrac distance matrices with resulting PCoA plots being visualised using the King viewer (Chen et al. 2009). For Illumina MiSeq 16s rRNA amplicon sequence data, three hundred base pair paired-end reads were assembled using FLASH (fast length adjustment of short reads to improve genome assemblies). Paired-end reads were further processed with the inclusion of quality filtering based on a quality score of  $> 25$  followed by subsequent removal of mismatched barcodes and sequences below length threshold using QIIME. USEARCH v7 (64-bit) was then used for denoising and chimera detection as well as clustering into operational taxonomic units (OTUs). PyNAST was used to align OTUs and taxonomy was assigned by using BLAST against the SILVA SSURef database release 111. QIIME

was used to generate Alpha and Beta diversities, calculated based on Bray Curtis, weighted and unweighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were then visualised using EMPeror v0.9.3-dev.

The nonparametric Kruskal-Wallis test was employed from within the Minitab (ver. 15, State College, PA, USA) statistical package to determine statistical significance between nonparametric data where significance was set at  $p \leq 0.05$ . Predictive functional profiling was performed using the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) tool (Langille et al. 2013) where again statistical significance was determined using the Kruskal-Wallis test with significance taken as  $p \leq 0.05$ .

### 3.4 RESULTS

#### 3.4.1 Undigested BSA does not invoke changes to the gut microbiota in an *ex vivo* distal colon model.

*Ex vivo* and *in vivo* studies were carried out to determine the impacts of specific whey proteins on the composition of the gut microbiota. Due to the nature of the *ex vivo* studies, i.e. fresh fecal samples were collected on the morning of each experiment and thus each experiment commenced with a different inoculating microbiota, statistical analysis of the sequence results was not possible and, thus, the focus was on identifying general patterns of change. The composition of an *ex vivo* distal colon microbiota, to which BSA was added at 0, 2.5% (5g BSA) and 5% (10g BSA), was investigated through the collection of samples at 0 (i.e. prior to the addition of BSA) and 24h and sequencing of the V4-V5 16s rRNA amplicons generated.  $\beta$ -diversity, based on unweighted Uifrac distances (Supplementary figure 1), did not show distinct clustering of the microbiota from the control (no BSA), 2.5% BSA or 5% BSA-containing samples. Taxonomic data was examined to find consistent alterations to individual populations across all three fermentation experiments. To this end, only *Prevotella* was observed to display a consistent pattern (figure 1). More specifically, the proportions of *Prevotella* decreased such that, *Prevotella* was not detected at the 24h end point from any of the control vessels, but was retained in vessels containing 2.5% (average 33% of 0h levels) or 5% (average 34% of 0h levels) BSA.

### **3.4.2 Digested lactoferrin and modified forms thereof are rapidly digested in an *in vitro* adult gastro-intestinal model.**

Lf-FOS Maillard reaction products (MRPs) were characterized in a previous work (Moscovici et al. 2014). It was chosen to further study the longest heating duration of 24h, to elucidate the effects of thermal processing without and with FOS, the latter leading to the formation of MRPs, on digestive fate throughout the gastrointestinal tract. Based on SDS-PAGE of digesta collected from an *in vitro* adult gastro-intestinal model (Figure 2), Lf is highly susceptible to *in vitro* adult gastric digestion even following thermal processing, as previously shown (Moscovici et al. 2014). The Lf band at ~80kDa is barely observed after 5min of gastric digestion in the native Lf sample (nLf) (Figure 2A). The Lf band is not observed at G5 in both samples of heated Lf (hLf) and Maillard (mLf) (Figure 2B and 2C, respectively), however, the proteolysis seems slower in mLf compared to hLf, as evident by the peptide breakdown profile. Nevertheless, based on the SDS-PAGE analysis, no significant differences were observed between nLf, hLf and mLf at the end of the gastro-intestinal digestion, which served as the "feeding material" for the *ex-vivo* distal colon model.

### **3.4.3 Digested lactoferrin and modified forms thereof have a similar impact on the $\beta$ diversity of an *ex vivo* distal colon microbiota.**

Calculated alpha diversity values showed an overall reduction in microbial diversity throughout the 24 hour experiment but that there were no differences with respect to the net impact of the lactoferrin variants on  $\alpha$ -diversity (Figure S2). Scatter plot analysis highlighted that the FOS containing vessel had the highest alpha diversity values at the 24 h time point.  $\beta$ -diversity (based upon unweighted unifrac distances)

showed a clear clustering by experimental run, and thus initial differences in the composition of the inoculating microbiota had a significant influence in this regard. When  $\beta$ -diversity was examined by time point (Figure 3), a tight clustering was observed between all treatments groups (FOS, nLf, hLf and mLf) at 0h after fecal slurry inoculation. As the fermentation progressed, PCoA analysis showed that all groups remain closely clustered at the 4 and 8 hour time points. At the 24h time point the nLf, hLf and mLf groups remain clustered tightly together and away from all other time points while the FOS control remains clustered in close proximity to the 4 and 8h points. This cluster pattern was observed across all three experimental runs.

Model distal colon microbiota inoculated with 1% pre-digested nLf, hLf and mLf have taxonomic profiles which are similar, but which differ from microbiota inoculated with 1% FOS

Immediately after fecal slurry inoculation, the bacterial profiles of all the groups in the experiment were the same with Firmicutes and Bacteroidetes being the dominant phyla (Figure S3). As the fermentation proceeded, the anticipated enrichment in the Actinobacteria phylum (and, more specifically *Bifidobacteriaceae/Bifidobacterium*) became apparent in the FOS control vessel for all three experimental runs (Supplementary figure 3a and figure 4). This was not observed in the nLf, hLf or mLf containing fermentation vessels. An increase in the proportions of the Proteobacteria phylum was observed from 4 hours after fecal slurry inoculation in all fermentation vessels (Figure S3a). This increase was notably higher in the nLf, hLf and mLf-containing vessels. By 24h Proteobacteria populations showed an average increase of 1284% (FOS), 2914% (nLf), 2197% (hLf) and 2563% (mLf).

Furthermore, proportions of the Proteobacteria-associated *Enterobacteriaceae/Escherichia-Shigella* (family/genus) were notably increased from 0 to 4 hours of fermentation in all vessels (Figure 4). However, this increase was observed to a considerably higher degree in the nLf, hLf and mLf containing vessels throughout the remainder of the fermentation period. *Bacteroidaceae/Bacteroidetes* populations remained consistent throughout the fermentation in the FOS containing vessel but decreased considerably in the nLf, hLf and mLf containing vessels from 8 to 24 hours of fermentation. Relative abundances of representatives of the family *Veillonellaceae* were noted to steadily increase in the nLf, hLf and mLf containing vessel resulting in a dramatic increase in overall proportions of this family between the 0h and 24h time points. This pattern was not observed in the FOS containing vessel. The associated genera *Acidominococcus* and *Phascolarctobacterium* followed a similar pattern in that they increased in each of the lactoferrin containing vessels from 8h to 24h of fermentation but not in the FOS control vessel. Figure 4 and Figure S3b provide a visual representation of the total assigned genus and family level taxonomies, respectively, for each of the three fermentation periods.

#### **3.4.4 Replacement of casein as the protein source of a high fat murine diet with either Lf or BSA alters $\beta$ diversity but not $\alpha$ diversity of the C57BL/6 mice**

McManus *et al.* conducted a study to investigate the effect of four different diets (HFD, LFD, HF-Lf and HF-BSA) on energy balance in C57BL/6 mice (McManus *et al.* 2015, McManus *et al.* 2015). Fecal pellets were taken from the same mice after 12 weeks of consuming the set diets to investigate, by high throughput DNA sequencing, their impact on the composition of the gut microbiota  $\alpha$ -diversity values



for biodiversity (Shannon index), observed species and species number relative to abundance within the sample (Simpson diversity index), number of observed species and species richness (Chao1). After 12 weeks of consuming set diets, no significant differences were established between any of the four diet groups for any of the measured alpha diversity indices (Figure 5).  $\beta$ -diversity (built upon unweighted unifracs distances) of the generated 16s rRNA sequences highlighted taxonomic differences between the gut microbiotas of animals consuming the LF, HF, HF-BSA and HF-Lf diets (Figure 6). PCoA analysis revealed a separation in clustering between the animals consuming the LF diet and those consuming the HF diet. Sequence data from animals who had consumed the HF-BSA and HF-Lf diets also showed a distinct clustering from each other as well from both the LF and HF diet consuming cohorts.

#### **3.4.5 Replacement of casein with either Lf or BSA in a high fat murine diet reverses several of the high fat associated shifts in microbial taxonomy**

Several of the significant taxonomic differences observed between the HFD and LFD cohorts were noted to be reversed upon the replacement of casein in the high fat murine diet with either Lf or BSA. At phylum level, proportions of Firmicutes were significantly higher in the HFD cohort when compared with either the LFD ( $p=0.027$ ), HF-Lf ( $p=0.005$ ) or HF-BSA ( $p=0.027$ ) cohorts (Figure 7A). Conversely, *Deferribacteres* populations were determined to be significantly higher in the LFD ( $p=0.021$ ) and HF-Lf ( $p=0.036$ ) groups compared to the HFD group.

Proportions of *Alcaligenaceae* (family) and *Parasutterella* (genus) were significantly higher in the LFD ( $p=0.004$ ), HF-Lf ( $p=0.011$ ) or HF-BSA ( $p<0.001$ ) cohorts when compared with the HFD cohort (figure 7B and 7C). Both

*Deferribacteraceae* and *Mucispirillum* were also significantly higher in the LFD ( $p=0.021$ ) and HF-Lf ( $p=0.036$  and  $p=0.016$ , respectively) cohorts. Other significant differences observed between the HFD and LFD groups included significantly higher proportions of the *Bacteroides* ( $p=0.036$ ), *Lachnospiraceae Incertae Sedis* ( $p=0.046$ ), *Catabacter* ( $p=0.020$ ) and *Anaerotruncus* ( $p=0.036$ ) genera in the LFD group compared to the HFD group, while the HFD cohort contained significantly higher populations of the *Peptostreptococcaceae Incertae Sedis* ( $p=0.028$ ) and *Akkermansia* ( $p=0.027$ ) genera compared to that of the LFD cohort (Figure 7).

Replacement of casein as the protein source of a high fat murine diet with either Lf or BSA also resulted in significant alterations to other taxonomic populations. At phylum level, a significant increase in the Proteobacteria populations were observed within the microbiotas of the HF-Lf ( $p=0.006$ ) and HF-BSA ( $p=0.016$ ) groups compared to the HFD group (figure 7A). Bacteroidetes proportions were also higher in the HF-Lf group ( $p=0.027$ ) when compared with the HFD group. At family level (figure 7B), *Streptococcaceae* proportions were determined to be significantly lower in both the HF-Lf and HF-BSA groups than either the HFD or LFD groups ( $p=0.001$ ) and *Anaeroplasmataceae* proportions were significantly lower in the HF-Lf group when compared to the HFD group ( $p=0.011$ ). Genus level taxonomic analysis revealed (figure 7C) that both HF-Lf and HF-BSA diet groups had significantly lower *Lactococcus* populations than either the LFD group ( $p=0.001$ ) or the HFD group ( $p=0.004$ ). Notably, the microbiota of the HF-Lf diet fed animals contained significantly higher levels of *Roseburia* compared to animals consuming the LFD ( $p=0.021$ ), HFD ( $p=0.012$ ) or the HF-BSA diet ( $p=0.014$ ) (Figure 7C).

Furthermore, several statistically significant differences in microbial taxonomy were observed between the gut microbiotas of animals consuming the HF-BSA and HF-Lf

diets. Animals consuming the HF-Lf diet had significantly higher proportions of *Bacteroidaceae* ( $p=0.046$ ), *Lachnospiraceae* ( $p=0.027$ ) and *Peptococcaceae* ( $p=0.016$ ) at family level and *Bacteroides* ( $p=0.046$ ) at genus level compared to those animals consuming the HF-BSA diet. Conversely, proportions of the genus *Oscillibacter* ( $p=0.027$ ) were significantly higher in the animals consuming the HF-BSA compared those provided with the HF-Lf diet. Figure 7 provides a visual representation of the statically significant taxonomic differences while Table S1 represents the relative abundance of all microbial taxonomies detected.

#### **3.4.6 PICRUSt analysis revealed that the metabolic potential of the HF-Lf and HF-BSA gut microbiota more closely resembles that of the LFD, than the HFD, mice.**

When compared to metabolic pathway scores assigned by PICRUSt within the HFD group, 44 and 27 pathways were determined to be significantly different within the HF-Lf and HF-BSA groups, respectively. Notably, only 7 metabolic pathways significantly differed in their frequencies between the LFD group and the HF-Lf group and no differences were determined between the LFD group and the HF-BSA group. Of the significantly altered metabolic pathways, glycolysis/gluconeogenesis and tyrosine metabolism were determined to be significantly higher in the HFD group than any of the other dietary groups ( $p<0.05$ ) while conversely, valine, leucine and isoleucine degradation was higher in all other diet groups compared to the HFD group ( $p<0.05$ ). A summary of all significantly altered metabolic pathways are summarised in Table 1.

### 3.5 Discussion

To date the effect of dietary proteins on the composition of the microbiota has not been extensively studied. It is clear, however, that protein fermentation is associated with a more diverse metabolite profile than other dietary macronutrients such as carbohydrates (Hamer et al. 2012) and that several of these metabolites play important roles in gut homeostasis, such as the formation of SCFAs and providing nitrogen for the growth of saccharolytic bacteria (Cummings and Macfarlane 1991, Conlon and Bird 2014). However, other metabolites resulting from microbial protein fermentation such as N-nitroso compounds, amines and p-cresol are potentially toxic or carcinogenic and thus a saccharolytic gut environment has been favoured over a proteolytic one (Gill and Rowland 2002, Rastall and Gibson 2015). This study examined the impact of the whey proteins BSA and Lf on the composition of the gut microbiota as assessed using an *ex-vivo* human and *in vivo* C57BL/6 murine models.

Anaerobic batch fermentation systems allow for the primary analysis of the impact of compounds of interest on the human gut microbiota. Addition of either 2.5% or 5% BSA into the batch fermentation system did not result in dramatic alterations to the gut microbial populations. One notable feature was the greater retention of the *Prevotella* genus within the BSA inoculated vessels which was not observed within the control vessel (Figure 1). Certain members of the *Prevotella* genus, such as *Prevotella ruminicola*, have been associated with protein fermentation owing to the highly active dipeptidyl peptidases and dipeptidases which they produce (Wallace 1996) and, thus, be more likely to benefit from the provision of BSA than other taxa.

Inoculation of the colonic fermentation system with Lf resulted in a similar taxonomic profile regardless of the pre-processing process that the Lf was exposed

to (i.e. thermal processing (hLf) or conjugation with FOS (mLf)) which differed from that achieved by FOS inoculation. This was also in agreement with SDS-PAGE results of gastro-intestinal proteolysis (Figure 2). As fermentation proceeded, a clear enrichment in the proportions of *Veillonellaceae*, *Acidominococcus* and *Phascolarctobacterium* (Figure 4 and Figure S3b) was observed in the vessels containing nLf, hLf or mLf but not within the FOS containing vessel. These bacterial groups have been shown to preferentially ferment amino acids and possess only limited saccharolytic activity, however they may contribute to improved gut health through the production of the SCFA propionate which can influence several cellular processes such as intestinal inflammation (Macfarlane and Macfarlane 1997, Arpaia et al. 2013, Scott et al. 2013, Smith et al. 2013). The *Bacteroides* genus is the dominant saccharolytic genus within the gut microbiota due to the large array of carbohydrate utilisation enzymes encoded within it (Salyers et al. 1977, Salyers et al. 1977, Xu et al. 2003, Sonnenburg et al. 2010). The present fermentation profile resulted in stable proportions of *Bacteroides* within the FOS containing vessel throughout the fermentation period (Figure 4), which is consistent with the ability of members of this genus to utilise FOS as a growth source (Endo et al. 2012). However, the dramatic decrease in the *Bacteroides* proportions observed within the Lf containing vessels between the 8 and 24 sampling points likely due to carbohydrate exhaustion, allowing for the enrichment of the aforementioned amino acid fermenting bacterial groups.

As expected (Kaplan and Hutkins 2000, Rossi et al. 2005, Russell et al. 2011, Flint et al. 2012), proportions of *Bifidobacterium* were observed to steadily increase throughout the fermentation period in the FOS containing vessel (Figure 4) due to its prebiotic properties. However, although Lf has been associated with playing an

important role in the establishment of the *Bifidobacterium* dominated infant gut microbiota and has also been shown to stimulate the growth of several species within the genus (such as *Bifidobacterium breve*) *in vitro* (Petschow et al. 1999, Kim et al. 2004, Mastromarino et al. 2014), no such enrichment was evident in the *ex vivo* model containing either nLf, hLf nor mLf. This may be as a consequence of the use of a more complex adult gut microbiota or the possibility that higher concentrations of Lf are required to invoke a bifidogenic effect.

Moreover, Lf is a natural defence protein possessing potent antimicrobial activity, driven mainly through two separate mechanisms. Firstly, Lf invokes bacteriostatic effects on enteric pathogens through its ability to sequester iron thus depriving microorganisms of this vital nutrient (Arnold and Cole 1977, Arnold et al. 1980). The second mechanism by which Lf invokes antimicrobial activity is by the direct interaction of the Lf molecule with the bacterial cell surface (Kalmar and Arnold 1988, Bortner et al. 1989, Farnaud and Evans 2003). Furthermore, pepsin digestion of Lf produces low molecular weight peptides known as lactoferricin (Lfcin) and Lactoferrampin (Lfampin) which show antimicrobial activity against a wide range of both gram positive and gram negative bacteria. Indeed Lfcin and Lfampin have a greater antimicrobial potency than native Lf (Bellamy et al. 1992, van der Kraan et al. 2004, Van der Kraan et al. 2006). In the present study, a notable increase in the relative abundance of a subset of the Proteobacteria phylum which corresponded to *Enterobacteriaceae* at family level and *Escherichia-Shigella* at genus level was observed in each of the Lf containing vessels compared to the FOS containing equivalent. This result suggests that a higher concentration may be required for Lf to invoke a beneficial modulatory effect on the adult gut microbiota, as previous studies have shown Lf (and Lf associated peptides) to display antimicrobial activity

against several members of the *Escherichia-Shigella* bacterial genus (Bellamy et al. 1993, Shin et al. 1998, Gomez et al. 2002).

It is now widely accepted that diet has a significant impact on gut microbiota composition. This relationship was apparent yet again here in that mice consuming a LFD, HFD, HF-Lf diet or HF-BSA diet for 12 weeks differed significantly with respect to their gut microbiota with beta diversity analysis based upon unweighted Unifrac distances revealing a clustering according to diet. Consistent with data generated from the *ex vivo* study of Lf on the composition of the adult human gut microbiota, switching the HFD protein source from casein to either Lf or BSA significantly increased the populations of Proteobacteria. As well as being associated with enteric inflammatory diseases (as stated above), increase in the Proteobacteria populations may also be detrimental to gut health due to their formation of N-nitroso compounds (NOCs) which can exert carcinogenic effects via DNA alkylation (Rowland 2000, Gill and Rowland 2002, Windey et al. 2012). The significant increase in Proteobacteria proportions observed in both the *ex vivo* and *in vivo* studies suggests that Lf (or associated peptide(s)) promotes increased populations of Proteobacteria within the gut microbiota and thus the antibacterial activity of Lf (at concentrations used presently) against members of the Proteobacteria phylum observed in *in vitro* studies (Shin et al. 1998, Gomez et al. 2002) does not appear to occur in the complex enteric microbial ecosystem.

As with other murine studies, high fat feeding significantly increased levels of Firmicutes while populations of *Bacteroidaceae* family and *Bacteroides* genus were significantly decreased compared to those animals in receipt of a LFD (Ley et al. 2005, Turnbaugh et al. 2006, Turnbaugh et al. 2008, Murphy et al. 2010, Daniel et al. 2014). Interestingly, substitution of casein with either Lf or BSA reversed several

of the HFD associated taxonomic shifts and enriched bacteria previously associated with the lean phenotype. Consistent with phylum level differences observed between the low and high fat diet groups, animals which consumed the HF-Lf and HF-BSA diets also had significantly lower populations of Firmicutes than the HFD group and significantly higher Bacteroidetes populations, in the case of the HF-Lf group. Notably, the inversion in the Firmicutes:Bacteroidetes ratio achieved by both LF and BSA compared to casein may in turn have beneficial implications in terms of weight gain and obesity (Ley et al. 2006). Mice consuming the HF-Lf or HF-BSA diets had significantly increased proportions of the *Alcaligenaceae* family and the *Parasuterella* genus compared to those consuming the HFD. An increase in populations of *Alcaligenaceae* was previously reported by Clarke *et al.* and Murphy *et al.* in lean mice compared to their diet-induced obese (DIO) counterparts, and *Alcaligenaceae* proportions within the DIO cohort were significantly increased after vancomycin treatment which was accompanied with improved metabolic function and reduced weight gain (Murphy et al. 2010, Clarke et al. 2013). A further study by Zhang *et al.* showed a significant increase in *Parasuterella* upon switching DIO C57BL/6 mice to a normal chow diet after 12 weeks of high fat diet feeding, a switch which restored overall structure, composition and bacterial diversity (Zhang et al. 2012).

In the context of alterations to the host phenotype reported by McManus *et al.* (McManus et al. 2015, McManus et al. 2015), the authors reported a significant decrease in body weight in the HF-BSA fed animals compared to that of the HFD fed animals which was associated with increased lean mass and reduced subcutaneous fat mass. Given the negligible effect of BSA on microbial populations observed in the *ex vivo* study, changes to microbial taxonomies observed presently



between the HFD and HF-BSA may result from the significant reduction in weight which has been shown in other studies to alter gut microbiota composition (Ley et al. 2006, Ravussin et al. 2012). Conversely, animals consuming the HF-Lf diet showed no difference in either body weight or body composition when compared to the animals consuming the HFD. High throughput sequence data generated presently therefore suggests that dietary Lf invokes changes to the gut HFD associated microbiota by a mechanism which is independent of host physiological state and therefore suggests a use for Lf as a beneficial modulator of the gut microbiota.

Mice in receipt of the HF-Lf diet displayed significantly higher levels of the *Roseburia* genus than mice on any of the other diet groups. Bacterial species contained within the *Roseburia* genus and other related genera, such as *Roseburia inulinivorans* and *Eubacterium rectale*, have been shown to be amongst the most dominant butyrate producers within the colonic ecosystem and thus are thought to be beneficial to the host (Aminov et al. 2006, Duncan et al. 2006, Louis and Flint 2009). Butyrate confers several health benefits to the host such as providing energy to colonocytes, reducing inflammation, exerting anti-carcinogenic effects and improving gut barrier function (Kruh 1981, Pryde et al. 2002, Flint et al. 2007, Peng et al. 2007). A reduced abundance of *Roseburia* has previously been linked with gastrointestinal (GI) inflammatory diseases such as Crohn's disease and ulcerative colitis (UC) which was associated with reduced butyrate levels. This data suggests that dietary Lf (or Lf associated bioactive peptides) may benefit host health by enriching growth of *Roseburia* either as a direct growth factor, or more likely as *Roseburia* depend on carbohydrates for growth (Duncan et al. 2007), by inhibition of competing bacterial species.

In line with observed taxonomic alterations to the gut microbiota, the PICRUST-based metabolic function analysis of *in vivo* data predicted several metabolic pathways to be significantly different between the HF-Lf and HF-BSA groups and the HFD group. Indeed, of the 251 predicted metabolic functions examined, the abundance of 44 pathways were significantly different in the HFD group compared to the HF-Lf group, while the abundance of 23 pathways were significantly different in the HFD group compared to the HF-BSA group. In contrast, the abundance of only 7 pathways were significantly different in the LFD group compared to the HF-Lf group and no differences were observed between the LFD group and the HF-BSA group. Within the microbiome of the HFD group, gluconeogenesis was predicted to be significantly higher than all other diet groups and have significantly higher fructose and mannose metabolism than that of the HF-Lf group. Notably, Turnbaugh *et al.* previously reported enrichment in both of these pathways in animals in receipt of a western diet suggesting that Lf and BSA may reduce the energy harvest of the gut microbiota on a high fat diet. Other significant differences were predicted between the LFD, HF-Lf and HF-BSA group compared to HFD group in that pathways associated with valine/leucine/isoleucine degradation and tyrosine metabolism were predicted to be decreased and increased, respectively, within the HFD microbiome. Valine, leucine and isoleucine can be oxidised by gut bacteria to form branched chain fatty acids (BCFAs) whose levels were reduced in patients with irritable bowel syndrome (IBS) and UC with respect to controls (Le Gall *et al.* 2011), while bacterial metabolism of tyrosine may result in the formation of cresols which are associated with a wide range of pathological conditions including those of the gut such as inflammatory bowel disease (IBD) (Nicholson *et al.* 2012). This data suggests that Lf and BSA may alter the functional potential of the gut microbiota to

reduce energy harvest from a high fat diet and may contribute to improved gut health.

The present study provides the first insight into the impact of the whey proteins bovine serum albumin and lactoferrin on the composition of the enteric microbial composition. The results, particularly those observed in C57BL/6 mice, suggest a use for these bovine whey proteins in the modulation of the gut microbiota to improve gut health which may reverse obesity and high fat feeding associated changes to microbial populations.

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**Table 1.** Statistically altered PICRUSt predicted metabolic pathways between C57BL/6 mice in receipt of a HFD, LFD, HF-Lf diet or HF-BSA diet.

Metabolic pathway	LFD	HFD	HF-Lf	HF-BSA
Processing;Membrane Transport;Bacterial secretion system	0.574 <sup>*</sup>	0.540	0.564 <sup>*</sup>	0.578 <sup>*</sup>
Processing;Membrane Transport;Transporters	6.894	7.488 <sup>¥†Δ</sup>	6.721	6.745
Processing;Signaling Molecules and Interaction;Bacterial toxins	0.140	0.147 <sup>†</sup>	0.132	0.138
Folding, Sorting and Degradation;Chaperones and folding catalysts	0.964 <sup>*</sup>	0.918	0.981 <sup>*</sup>	0.977 <sup>*</sup>
Folding, Sorting and Degradation;Protein processing in endoplasmic reticulum	0.073 <sup>*</sup>	0.062	0.073 <sup>*</sup>	0.074 <sup>*</sup>
Processing;Folding, Sorting and Degradation;RNA degradation	0.451 <sup>*</sup>	0.431	0.464 <sup>*</sup>	0.459 <sup>*</sup>
Processing;Transcription;Transcription factors	1.464	1.609 <sup>¥Δ</sup>	1.501	1.446
Amino Acid Metabolism;Phenylalanine, tyrosine and tryptophan biosynthesis	0.790 <sup>*</sup>	0.756	0.826 <sup>*</sup>	0.800 <sup>*</sup>
Amino Acid Metabolism;Tyrosine metabolism	0.315	0.335 <sup>¥†Δ</sup>	0.308	0.318
Amino Acid Metabolism;Valine, leucine and isoleucine degradation	0.263 <sup>*</sup>	0.253	0.270 <sup>*</sup>	0.263 <sup>*</sup>
Carbohydrate Metabolism;Citrate cycle (TCA cycle)	0.503	0.423	0.540 <sup>*</sup>	0.510
Carbohydrate Metabolism;Fructose and mannose metabolism	1.237	1.339 <sup>†</sup>	1.094	1.239
Carbohydrate Metabolism;Glycolysis / Gluconeogenesis	1.060	1.096 <sup>¥†Δ</sup>	1.049	1.067
Carbohydrate Metabolism;Pentose phosphate pathway	0.900	0.949 <sup>†</sup>	0.870	0.909
Energy Metabolism;Carbon fixation pathways in prokaryotes	0.905	0.816	0.952 <sup>*</sup>	0.912
Energy Metabolism;Nitrogen metabolism	0.652 <sup>*</sup>	0.632	0.656 <sup>*</sup>	0.661 <sup>*</sup>

PICRUSt analysis of 16s rRNA sequence data of metabolic profiles where a significant difference was observed between dietary groups. Data is expressed as the relative abundance of the metabolic pathway and where \* indicates a significantly higher abundance than the HFD group, ¥ indicates a significantly higher abundance than the LFD group, † indicates a significantly higher abundance than the HF-Lf group and Δ indicates a significantly higher abundance than the HF-BSA group. Statistical significance determined using the Kruskal-Wallis test where significance was taken as  $p \leq 0.05$ .

**Table 1. (Continued)**

<b>Metabolic pathway</b>	<b>LFD</b>	<b>HFD</b>	<b>HF-Lf</b>	<b>HF-BSA</b>
Glycan Biosynthesis and Metabolism;Glycosyltransferases	0.306 <sup>*</sup>	0.290	0.309 <sup>*</sup>	0.302
Glycan Biosynthesis and Metabolism;Lipopolysaccharide biosynthesis	0.225 <sup>*</sup>	0.151	0.224 <sup>*</sup>	0.229 <sup>*</sup>
Glycan Biosynthesis and Metabolism;Lipopolysaccharide biosynthesis proteins	0.312 <sup>*</sup>	0.232	0.311 <sup>*</sup>	0.319 <sup>*</sup>
Glycan Biosynthesis and Metabolism;N-Glycan biosynthesis	0.022	0.014	0.023 <sup>*</sup>	0.020
Lipid Metabolism;Glycerolipid metabolism	0.399	0.417 <sup>†Δ</sup>	0.394	0.390
Lipid Metabolism;Linoleic acid metabolism	0.078 <sup>†</sup>	0.083 <sup>†</sup>	0.068	0.076 <sup>†</sup>
Metabolism of Cofactors and Vitamins;Biotin metabolism	0.115	0.096	0.126 <sup>*</sup>	0.117
Metabolism of Cofactors and Vitamins;Folate biosynthesis	0.350 <sup>*</sup>	0.323	0.353 <sup>*</sup>	0.356
Metabolism of Cofactors and Vitamins;Pantothenate and CoA biosynthesis	0.507	0.475	0.562 <sup>*</sup>	0.513
Metabolism of Other Amino Acids;beta-Alanine metabolism	0.165	0.150	0.184 <sup>*</sup>	0.169
Metabolism of Terpenoids and Polyketides;Geraniol degradation	0.024	0.015	0.036 <sup>*</sup>	0.024
Biodegradation and Metabolism;Benzoate degradation	0.211	0.229 <sup>¥</sup>	0.225 <sup>¥Δ</sup>	0.210 <sup>*</sup>
Biodegradation and Metabolism;Bisphenol degradation	0.088	0.093 <sup>†Δ</sup>	0.079	0.086
Biodegradation and Metabolism;Caprolactam degradation	0.011	0.007	0.016 <sup>*Δ</sup>	0.008
Biodegradation and Metabolism;Chloroalkane and chloroalkene degradation	0.162 <sup>†</sup>	0.172 <sup>¥†</sup>	0.151	0.158
Biodegradation and Metabolism;Styrene degradation	0.008	0.009 <sup>Δ</sup>	0.014 <sup>¥Δ</sup>	0.007

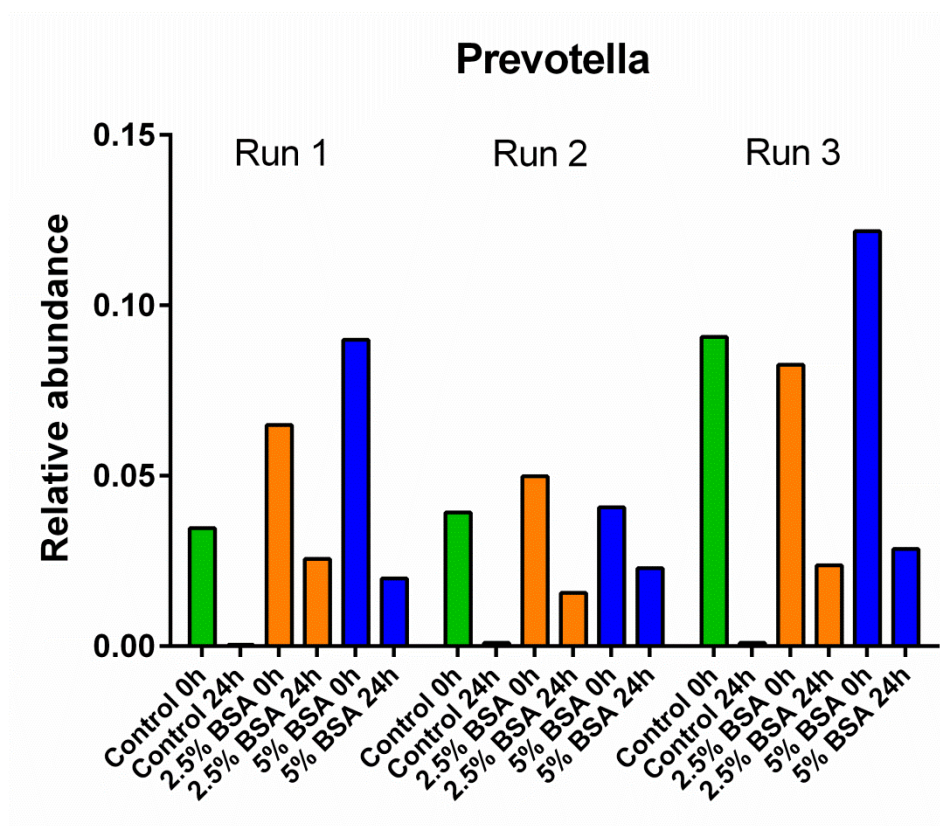
PICRUSt analysis of 16s rRNA sequence data of metabolic profiles where a significant difference was observed between dietary groups. Data is expressed as the relative abundance of the metabolic pathway and where \* indicates a significantly higher abundance than the HFD group, ¥ indicates a significantly higher abundance than the LFD group, † indicates a significantly higher abundance than the HF-Lf group and Δ indicates a significantly higher abundance than the HF-BSA group. Statistical significance determined using the Kruskal-Wallis test where significance was taken as  $p \leq 0.05$ .

**Table 1. (Continued)**

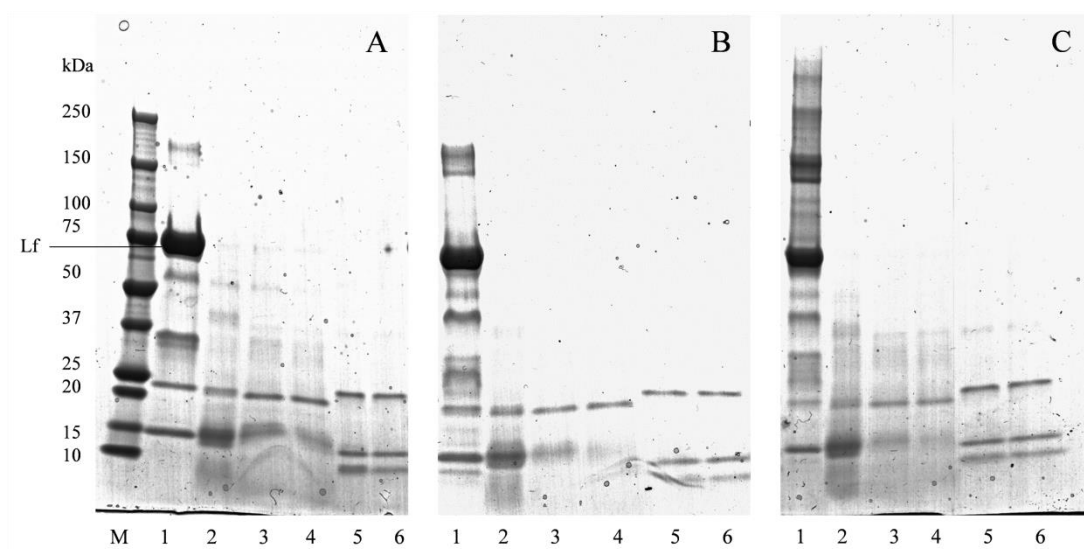
<b>Metabolic pathway</b>	<b>LFD</b>	<b>HFD</b>	<b>HF-Lf</b>	<b>HF-BSA</b>
Biodegradation and Metabolism;Toluene degradation	0.063 <sup>*</sup>	0.042	0.074 <sup>*</sup>	0.071 <sup>*</sup>
Endocrine System;Adipocytokine signaling pathway	0.047	0.035	0.049 <sup>*</sup>	0.051
Cellular Processes and Signaling;Cell motility and secretion	0.159	0.141	0.159 <sup>*</sup>	0.164 <sup>*</sup>
Cellular Processes and Signaling;Germination	0.013	0.012	0.023 <sup>*¥Δ</sup>	0.011
Cellular Processes and Signaling;Membrane and intracellular structural molecules	0.477 <sup>*</sup>	0.406	0.479 <sup>*</sup>	0.485 <sup>*</sup>
Cellular Processes and Signaling;Other transporters	0.245	0.255 <sup>¥†</sup>	0.243	0.252
Cellular Processes and Signaling;Pores ion channels	0.270	0.228	0.291 <sup>*</sup>	0.291
Genetic Information Processing;Protein folding and associated processing	0.708 <sup>*†</sup>	0.685	0.680	0.699
Metabolism;Amino acid metabolism	0.230	0.230 <sup>†</sup>	0.221	0.234
Unclassified;Metabolism;Others	1.017	1.080 <sup>¥†Δ</sup>	0.970	0.999

PICRUSt analysis of 16s rRNA sequence data of metabolic profiles where a significant difference was observed between dietary groups. Data is expressed as the relative abundance of the metabolic pathway and where \* indicates a significantly higher abundance than the HFD group, ¥ indicates a significantly higher abundance than the LFD group, † indicates a significantly higher abundance than the HF-Lf group and Δ indicates a significantly higher abundance than the HF-BSA group. Statistical significance determined using the Kruskal-Wallis test where significance was taken as  $p \leq 0.05$ .

**Figure 1.** Bar charts representing the relative abundance of the *Prevotella* genus in the control, 2.5% BSA and 5% BSA containing vessels at the 0 and 24h time points.

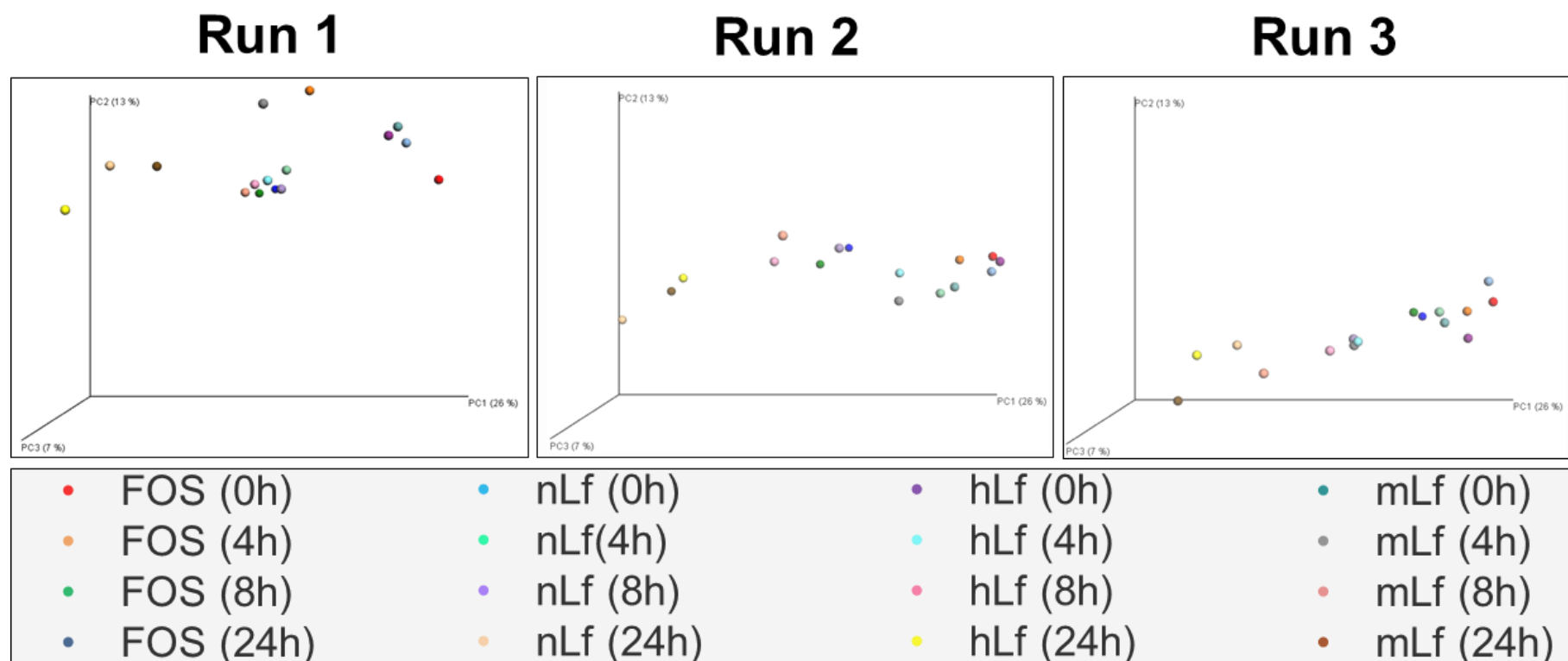


**Figure 2.** Comparison of peptide breakdown profiles of Lf digesta samples based on SDS-PAGE, where A) represents nLf, B) hLf and C) mLf.

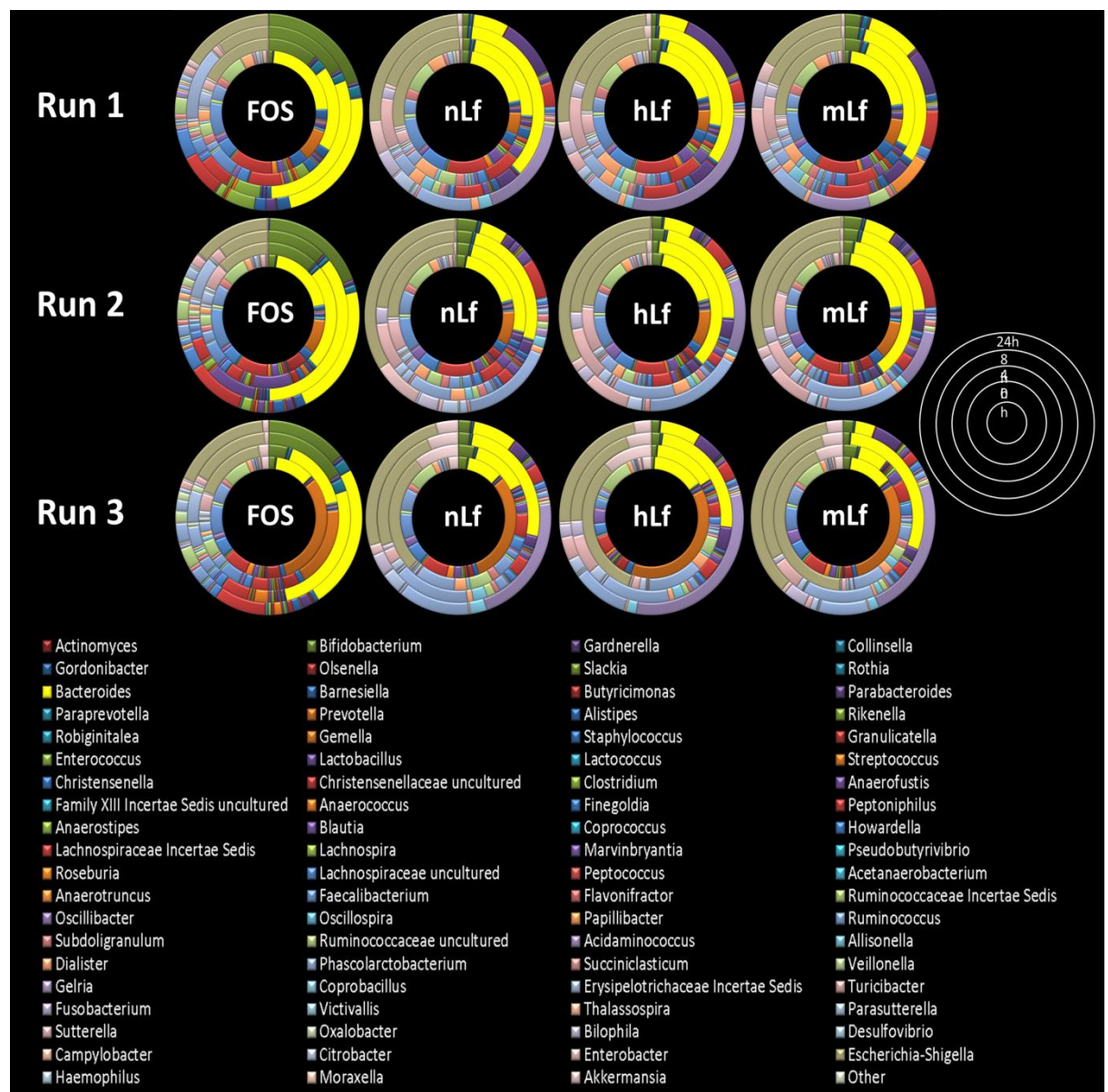




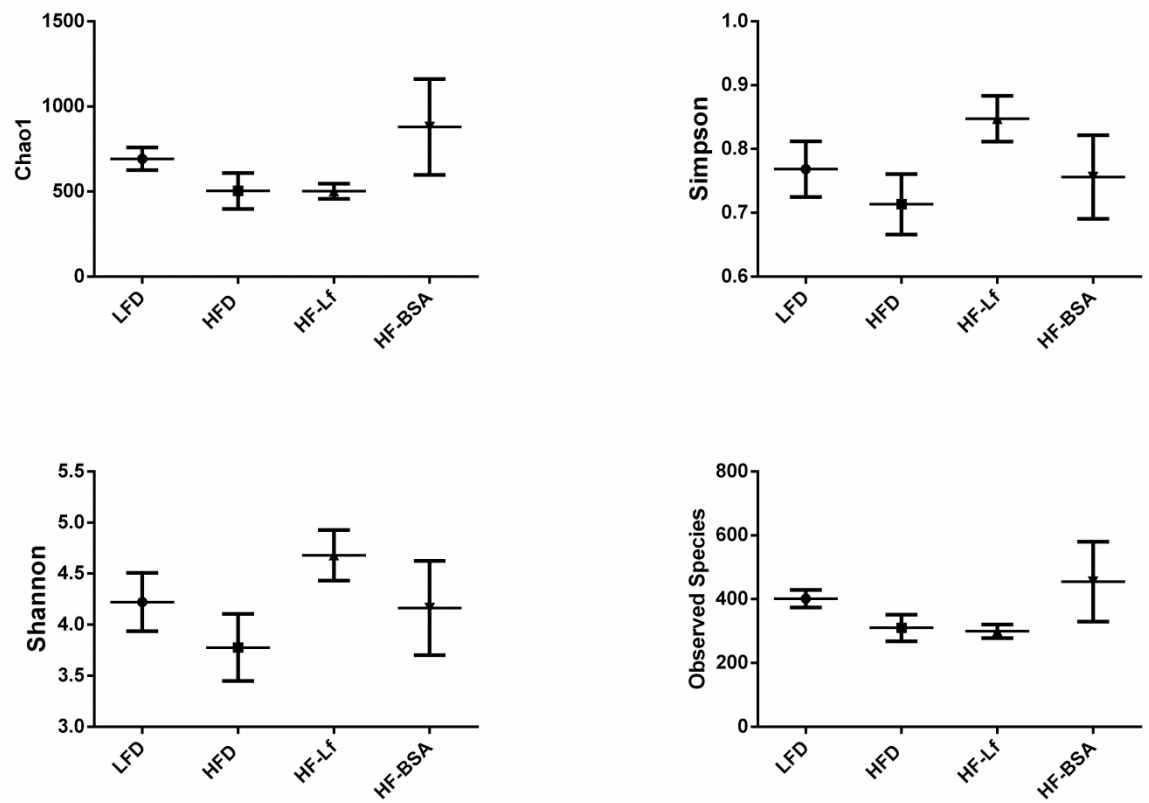
**Figure 3.** Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of generated 16s rRNA sequences highlighting the change in microbial composition along the 24h batch fermentation in response to addition of 1% FOS, nLf, hLf or mLf.



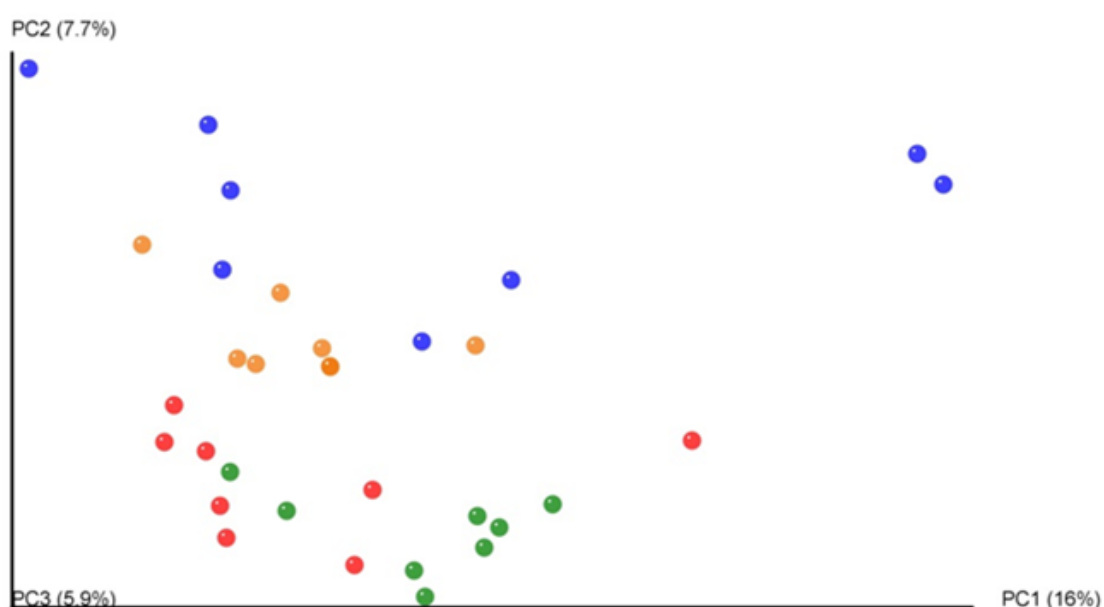
**Figure 4.** Doughnut charts representing the relative abundance of detected microbial taxonomies at genus level at 0, 4, 8 and 24h of a simulated colonic fermentation in response to the addition of 1% (w/v) FOS, nLf, hLf or mLf.



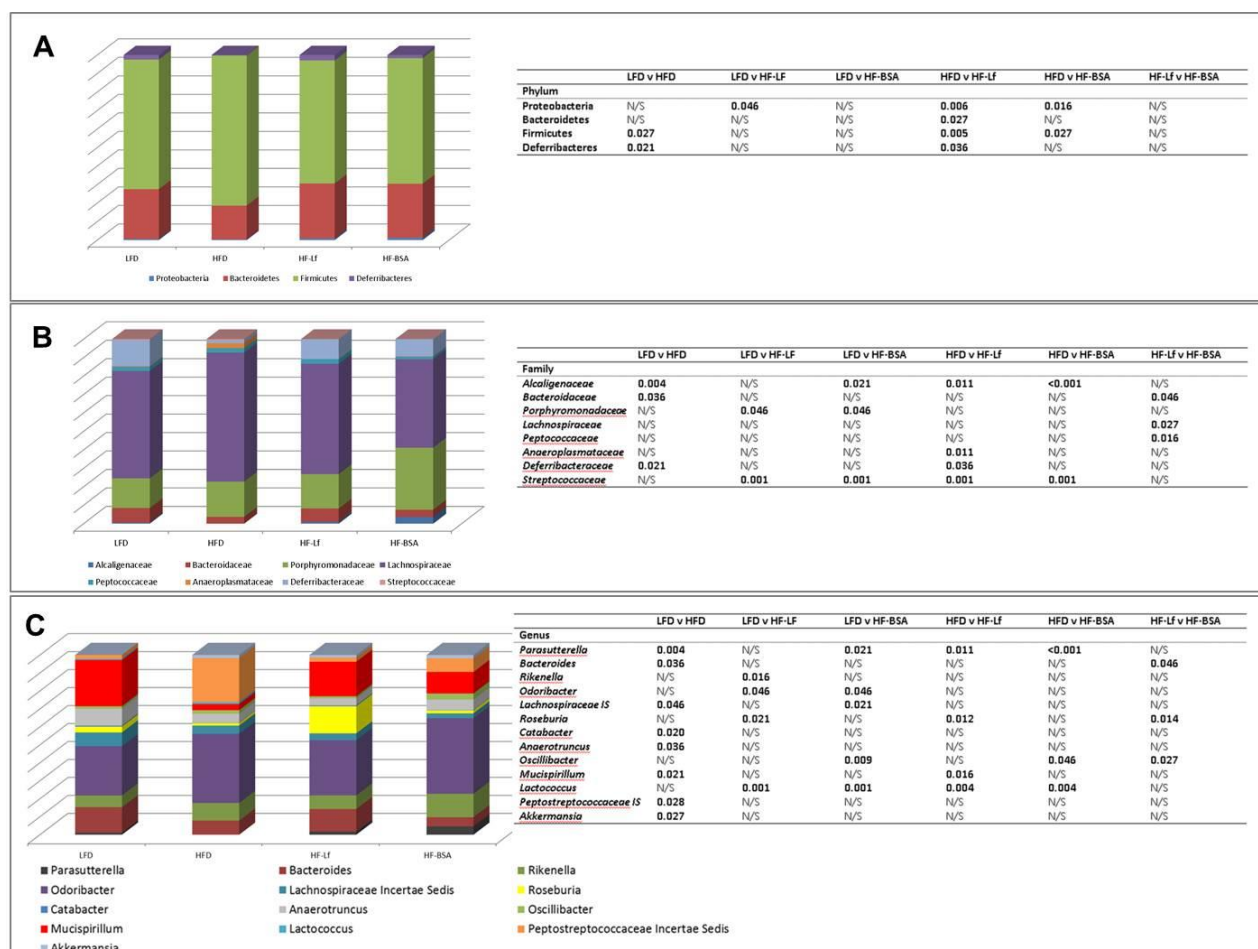
**Figure 5.** Comparison of alpha diversity indices across the HFD, LFD, HF-LF and HF-BSA consuming cohorts. Data shown are means  $\pm$  SEM (n=8).



**Figure 6.** Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of generated 16s rRNA sequences demonstrating a clustering according to diet group, where • represents sequence data generated from LFD cohort, • represents sequence data generated from the HFD cohort, • represents sequence data generated from the HF-Lf diet cohort and • represents sequence data generated from the HF-BSA diet cohort.



**Figure 7.** Stacked bar charts representing the significantly altered microbiota taxonomies at A) phylum, B) family and C) genus level within the gut microbiotas of C57BL/6 mice after 12 weeks of consuming either a HFD, LFD, HF-Lf diet or HF-BSA diet, with the corresponding p-values, as determined by the Kruskal-Wallis statistical test ( $p \leq 0.05$ ).



### 3.7 SUPPORTING INFORMATION

**Table S1.** Microbial taxonomies detected, by high-throughput pyrosequencing, within the gut microbiota of C57BL/6 mice consuming set diets for 12 weeks

	LF	HF	HF-Lf	HF-BSA
<b>Phylum</b>				
Proteobacteria	0.62	0.36	0.88 <sup>*¥</sup>	1.20 <sup>*</sup>
Bacteroidetes	27.33	17.78	31.09 <sup>*</sup>	34.47
Firmicutes	67.59	79.63 <sup>¥†Δ</sup>	63.46	61.01
Deferribacteres	2.26 <sup>*</sup>	0.32	2.32 <sup>*</sup>	1.53
Tenericutes	0.34	0.37	0.32	0.60
Verrucomicrobia	0.38	0.16	0.27	0.24
Actinobacteria	1.48	1.38	1.65	0.95
<b>Family</b>				
<i>Alcaligenaceae</i>	0.12 <sup>*</sup>	0.00	0.33 <sup>*</sup>	0.62 <sup>*¥</sup>
<i>Bacteroidaceae</i>	1.42 <sup>*</sup>	0.74	1.96 <sup>Δ</sup>	1.04
<i>Desulfovibrionaceae</i>	0.48	0.35	0.49	0.54
<i>Rikenellaceae</i>	8.22	6.83	9.38	12.26
<i>Porphyromonadaceae</i>	2.91	3.50	5.20 <sup>¥</sup>	7.76 <sup>¥</sup>

Microbial taxonomies detected within the microbiotas of C57BL/6 mice after 12 weeks consuming either a HF, LF, HF-Lf diet or HF-BSA diet. Data is expressed as the total percentage of bacteria present within the data set and where \* indicates a value significantly higher than the HFD group, ¥ indicates a value significantly higher than the LFD group, † indicates a value significantly higher than the HF-Lf group and Δ indicates a value significantly higher than the HF-BSA group, where significance was taken as  $p \leq 0.05$  as determined by the non-parametric Kruskal-Wallis test.

**Table S1. (Continued)**

	LF	HF	HF-Lf	HF-BSA
<i>Bifidobacteriaceae</i>	<b>1.43</b>	<b>1.32</b>	<b>1.55</b>	<b>0.84</b>
<i>Lactobacillaceae</i>	<b>4.85</b>	<b>6.78</b>	<b>5.48</b>	<b>3.16</b>
<i>Erysipelotrichaceae</i>	<b>44.80</b>	<b>49.68</b>	<b>31.75</b>	<b>37.04</b>
<i>Lachnospiraceae</i>	<b>9.78</b>	<b>13.00</b>	<b>15.91<sup>Δ</sup></b>	<b>9.10</b>
<i>Ruminococcaceae</i>	<b>6.62</b>	<b>5.93</b>	<b>7.65</b>	<b>7.94</b>
<i>Peptococcaceae</i>	<b>0.38</b>	<b>0.45</b>	<b>0.66<sup>Δ</sup></b>	<b>0.30</b>
<i>Anaeroplasmataceae</i>	<b>0.05</b>	<b>0.45<sup>†</sup></b>	<b>0.00</b>	<b>0.03</b>
<i>Deferribacteraceae</i>	<b>2.26</b>	<b>0.32</b>	<b>2.29<sup>*</sup></b>	<b>1.53</b>
<i>Coriobacteriaceae</i>	<b>0.05</b>	<b>0.04</b>	<b>0.09</b>	<b>0.10</b>
<i>Streptococcaceae</i>	<b>0.06<sup>†Δ</sup></b>	<b>0.11<sup>†Δ</sup></b>	<b>0.00</b>	<b>0.00</b>
<i>Rhodospirillaceae</i>	<b>0.01</b>	<b>0.00</b>	<b>0.02</b>	<b>0.02</b>
<i>Akkermansiaceae</i>	<b>0.38</b>	<b>0.16</b>	<b>0.27</b>	<b>0.24</b>
<i>Enterobacteriaceae</i>	<b>0.00</b>	<b>0.01</b>	<b>0.00</b>	<b>0.00</b>
<i>Clostridiaceae</i>	<b>0.00</b>	<b>0.34</b>	<b>0.06</b>	<b>0.00</b>

Microbial taxonomies detected within the microbiotas of C57BL/6 mice after 12 weeks consuming either a HF, LF, HF-Lf diet or HF-BSA diet. Data is expressed as the total percentage of bacteria present within the data set and where \* indicates a value significantly higher than the HFD group, ‡ indicates a value significantly higher than the LFD group, † indicates a value significantly higher than the HF-Lf group and Δ indicates a value significantly higher than the HF-BSA group, where significance was taken as  $p \leq 0.05$  as determined by the non-parametric Kruskal-Wallis test.

**Table S1. (Continued)**

	LF	HF	HF-Lf	HF-BSA
<b>Genus</b>				
<i>Parasutterella</i>	0.12 <sup>*</sup>	0.00	0.32 <sup>*</sup>	0.62 <sup>*Δ</sup>
<i>Desulfovibrio</i>	0.38	0.25	0.35	0.45
<i>Bilophila</i>	0.09	0.09	0.14	0.09
<i>Bacteroides</i>	1.42 <sup>*</sup>	0.74	1.96 <sup>Δ</sup>	1.04
<i>RC9 gut group</i>	2.40	1.69	2.04	2.07
<i>Alistipes</i>	5.21	4.25	6.12	8.05
<i>Rikenella</i>	0.60	0.88	1.21 <sup>¥</sup>	2.11
<i>Odoribacter</i>	2.73	3.41	4.94 <sup>¥</sup>	7.58 <sup>¥</sup>
<i>Parabacteroides</i>	0.17	0.39	0.25	0.18
<i>Bifidobacterium</i>	1.43	1.00	1.55	0.84
<i>Lactobacillus</i>	4.85	5.37	5.46	3.15
<i>Allobaculum</i>	44.79	49.58	31.66	37.03
<i>Lachnospiraceae IS</i>	0.72 <sup>*Δ</sup>	0.39	0.57	0.39
<i>Roseburia</i>	0.30	0.12	2.19 <sup>*¥Δ</sup>	0.21

Microbial taxonomies detected within the microbiotas of C57BL/6 mice after 12 weeks consuming either a HF, LF, HF-Lf diet or HF-BSA diet. Data is expressed as the total percentage of bacteria present within the data set and where \* indicates a value significantly higher than the HFD group, ¥ indicates a value significantly higher than the LFD group, † indicates a value significantly higher than the HF-Lf group and Δ indicates a value significantly higher than the HF-BSA group, where significance was taken as  $p \leq 0.05$  as determined by the non-parametric Kruskal-Wallis test.



**Table S1. (Continued)**

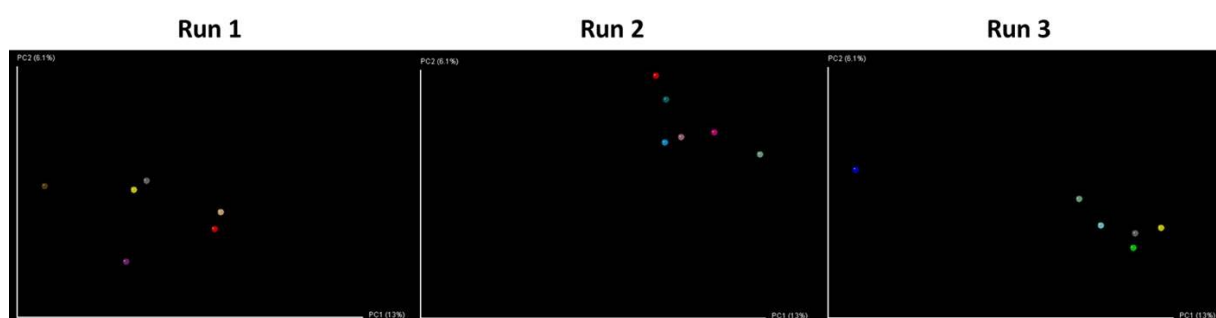
	LF	HF	HF-Lf	HF-BSA
<i>Coprococcus</i>	<b>0.14</b>	<b>0.10</b>	<b>0.89</b>	<b>0.27</b>
<i>Catabacter</i>	<b>0.05*</b>	<b>0.02</b>	<b>0.08</b>	<b>0.02</b>
<i>Ruminococcaceae IS</i>	<b>3.67</b>	<b>3.24</b>	<b>3.74</b>	<b>4.92</b>
<i>Anaerotruncus</i>	<b>0.90*</b>	<b>0.45</b>	<b>0.54</b>	<b>0.78</b>
<i>Oscillibacter</i>	<b>0.13</b>	<b>0.17</b>	<b>0.19</b>	<b>0.46*‡</b>
<i>Peptococcus</i>	<b>0.24</b>	<b>0.36</b>	<b>0.35</b>	<b>0.24</b>
<i>Anaeroplasma</i>	<b>0.43</b>	<b>0.05</b>	<b>0.08</b>	<b>0.03</b>
<i>RF9</i>	<b>0.29</b>	<b>0.32</b>	<b>0.26</b>	<b>0.57</b>
<i>Mucispirillum</i>	<b>2.26*</b>	<b>0.32</b>	<b>2.36*</b>	<b>1.53</b>
<i>Coriobacteriaceae</i>	<b>0.01</b>	<b>0.03</b>	<b>0.02</b>	<b>0.01</b>
<i>Lactococcus</i>	<b>0.06<sup>†Δ</sup></b>	<b>0.09<sup>†Δ</sup></b>	<b>0.00</b>	<b>0.00</b>
<i>Peptostreptococcaceae IS</i>	<b>0.16</b>	<b>2.13<sup>¥</sup></b>	<b>0.37</b>	<b>1.96</b>
<i>Thalassospira</i>	<b>0.00</b>	<b>0.00</b>	<b>0.02</b>	<b>0.02</b>
<i>Enterorhabdus</i>	<b>0.01</b>	<b>0.02</b>	<b>0.07</b>	<b>0.05</b>
<i>Akkermansia</i>	<b>0.00</b>	<b>0.16<sup>¥</sup></b>	<b>0.24</b>	<b>0.24</b>
<i>Turicibacter</i>	<b>0.00</b>	<b>0.08</b>	<b>0.02</b>	<b>0.00</b>
<i>Blautia</i>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.02</b>
<i>Ruminococcus</i>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.01</b>
<i>Uncultured bacteria</i>	<b>0.54</b>	<b>1.95</b>	<b>0.97</b>	<b>0.66</b>

Microbial taxonomies detected within the microbiotas of C57BL/6 mice after 12 weeks consuming either a HF, LF, HF-Lf diet or HF-BSA diet. Data is expressed as the total percentage of bacteria present within the data set and where \* indicates a value significantly higher than the HFD group, ¥ indicates a value significantly higher than the LFD group, † indicates a value significantly higher than the HF-Lf group and Δ indicates a value significantly higher than the HF-BSA group, where significance was taken as  $p \leq 0.05$  as determined by the non-parametric Kruskal-Wallis test.

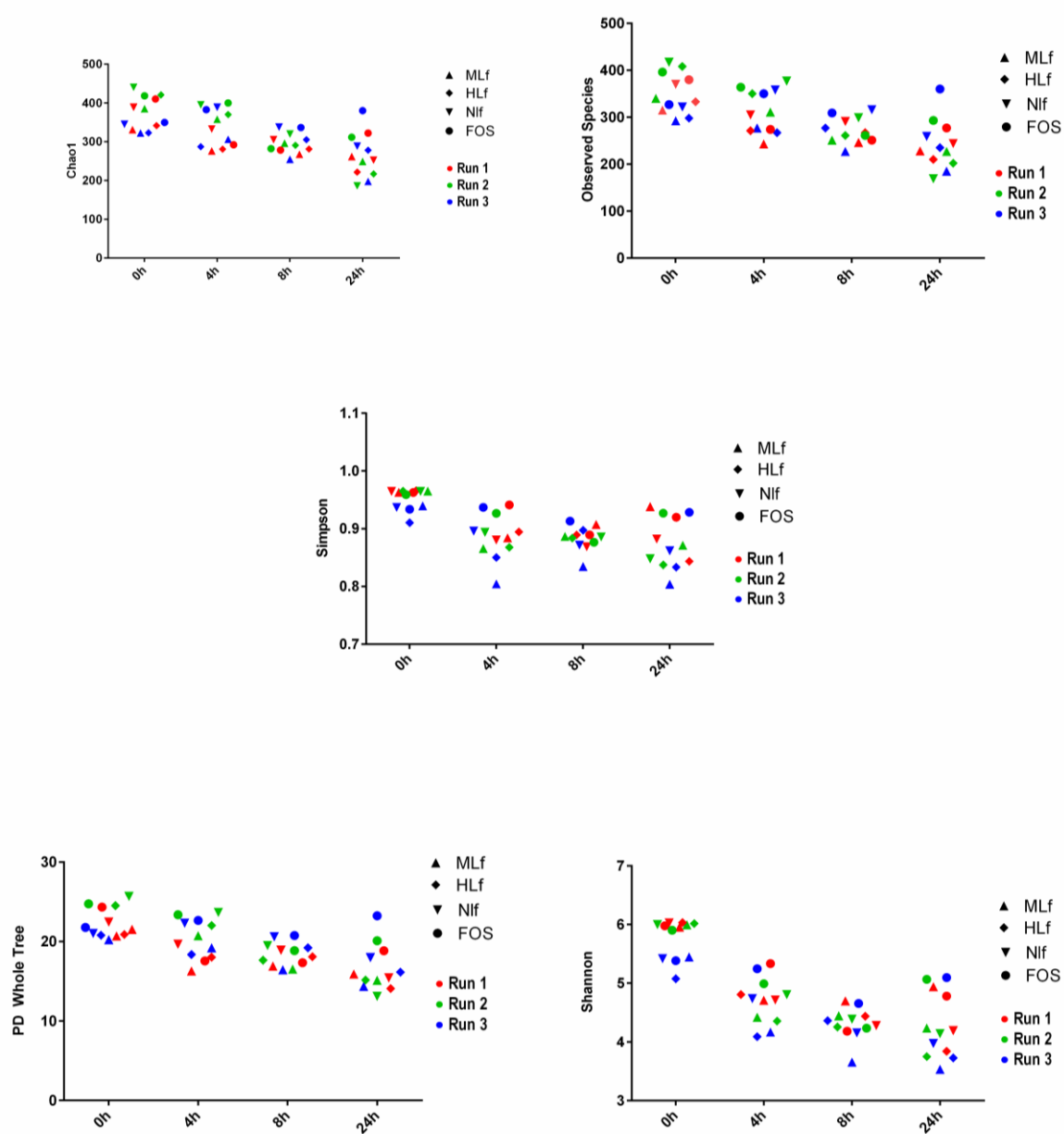
**Table S2.** Total 16s rRNA sequence reads generated by sequencing processes

Experiment	Sequencing platform	16s rRNA gene region	Total reads generated	Reads per sample
Impact of 2.5% and 5% BSA on the adult human gut microbiota in a distal colon model.	Roche 454 high-throughput pyrosequencing	V4-V5	182,890	10,161
Impact of nLf, hLf and mLf on the adult gut microbiota in a distal colon model.	Illumina MiSeq 16s rRNA amplicon sequencing	V3-V4	1,396,892	29,102
Investigation of the substitution of casein with either Lf or BSA in a high fat murine diet on the gut microbiota of C57BL/6 mice.	Roche 454 high-throughput pyrosequencing	V4-V5	371,333	11,604

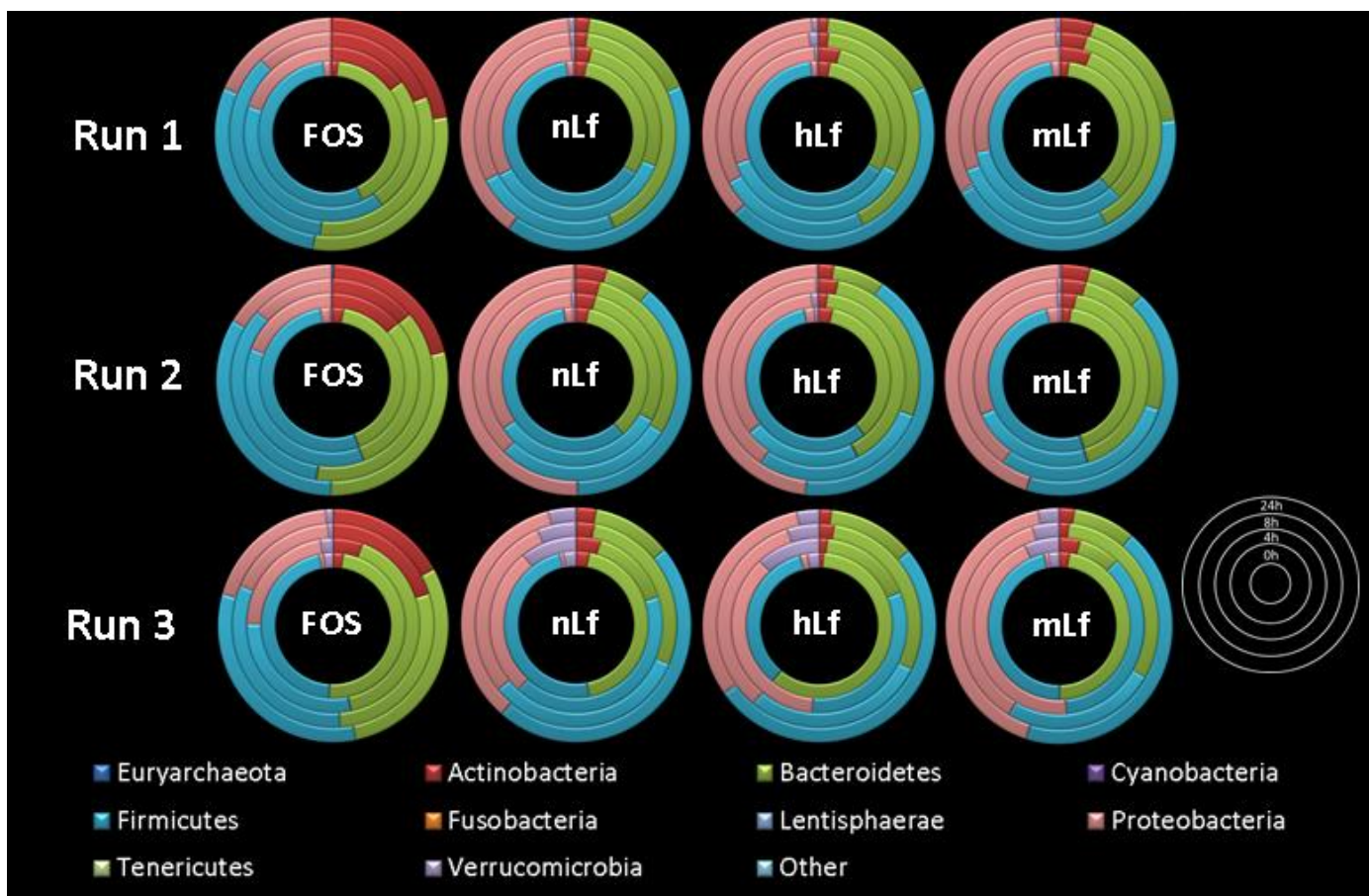
**Figure S1.** Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of generated 16S rRNA sequences across 3 runs of an *ex vivo* simulated adult human microbiota fermentation containing 2.5% BSA, 5% BSA or no additional BSA (control). Where, • represents Control (0h), • represents control (24h), • represents 2.5% BSA (0h), • represents 2.5% BSA (24h), • represents 5% BSA (0h) and • represents 5% BSA (24h).



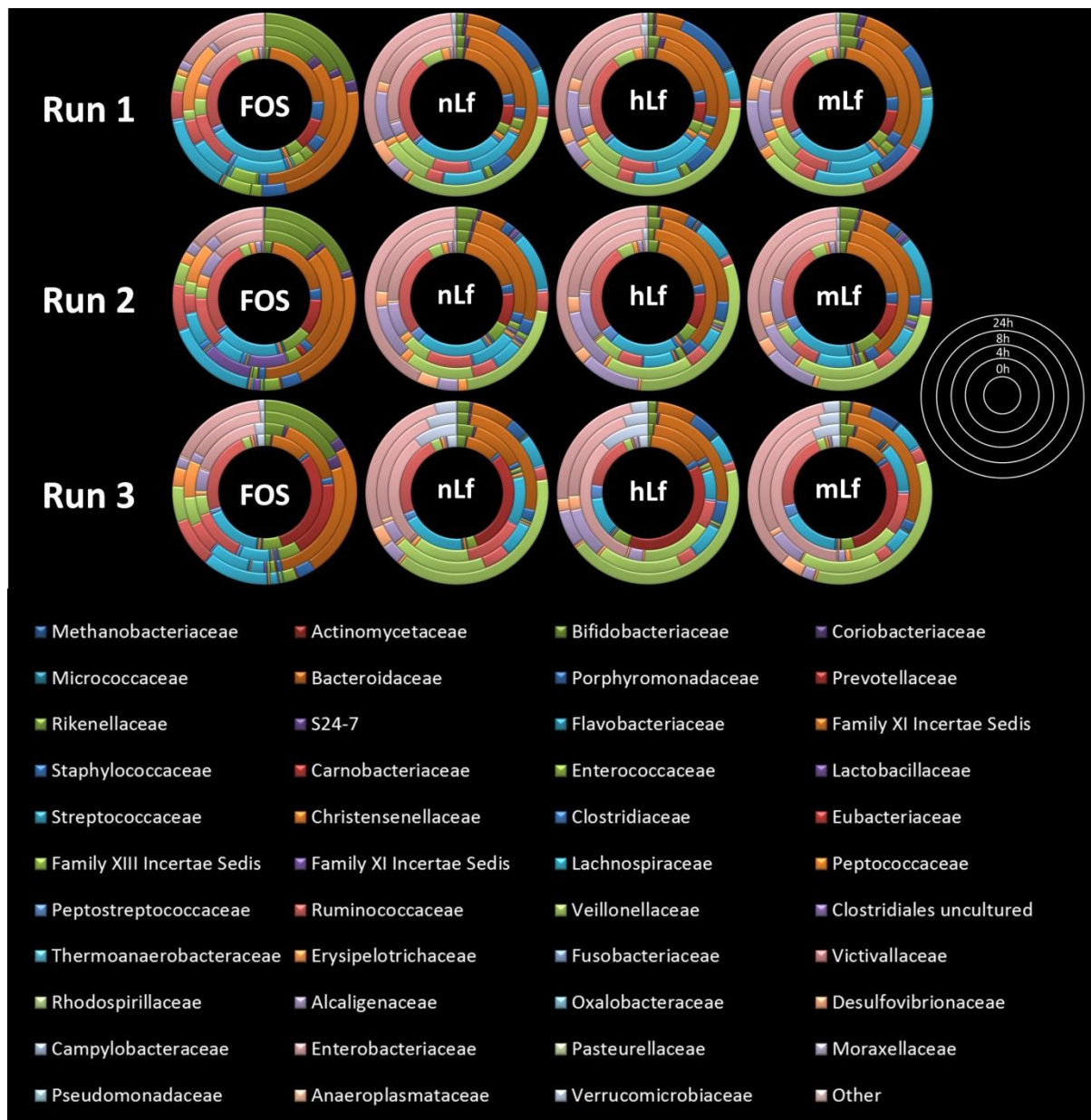
**Figure S2.** Scatter plot analysis representing the assigned score for each examined alpha diversity measure (Chao1, Simpson index, Shannon index, PD Whole Tree and observed species) at 0, 4, 8 and 24h of an *ex vivo* fermentation with an adult human gut microbiota with the addition of 1% (w/v) FOS, nLf, hLf or mLf.



**Figure S3a.** Doughnut charts representing the relative abundance of detected microbial taxonomies at phylum level at 0, 4, 8 and 24h of a simulated colonic fermentation in response to the addition of 1% (w/v) FOS, nLf, hLf or mLf.



**Figure S3b.** Doughnut charts representing the relative abundance of detected microbial taxonomies at family level at 0, 4, 8 and 24h of a simulated colonic fermentation in response to the addition of 1% (w/v) FOS, nLf, hLf or mLf.



## **Chapter 4**

**Voluntary exercise alters energy balance and the gut microbiota in a time dependent manner in C57BL/6 mice fed a low fat diet.**

**Manuscript in review**

## 4.1 ABSTRACT

Here, we investigated the effect of voluntary exercise on energy balance and related parameters, including the composition of the gut microbiota, in male C57BL/6 mice. Mice consumed a 10% energy (low) fat diet (LFD), with (RW+) or without (RW-) access to running wheels for eight weeks. The RW+ group had similar body weight trajectory and body composition to RW- animals, but consumed significantly more food by week three of the trial ( $p<0.05$ ), as a result of higher intake during the dark phase. Metabolic activity was increased in the RW+, both in the light and dark phases, compared to RW- group and respiratory exchange ratio was significantly increased in the dark phase in the RW+ compared to RW- group, indicating increased tissue carbohydrate metabolism ( $p<0.05$ ). This was supported by unchanged levels of plasma insulin and glucose and by a trend towards an increased expression of hypothalamic insulin receptor (INSR) ( $p=0.085$ ), glucose transporter 1 (Glut1) ( $p=0.085$ ), insulin receptor substrate 1 (IRS1) ( $p=0.065$ ), fatty acid synthase (FASN) ( $p=0.071$ ) and peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ) ( $p=0.052$ ), all at the trial's week 8 end point. High throughput DNA sequencing revealed subtle changes in the microbiota of the RW+ group relative to the RW- group. More specifically, at week 4 the RW+ cohort contained significantly higher proportions of *Bacteroidaceae/Bacteroides* and significantly lower levels of *Clostridiaceae/Clostridium* compared to the RW- cohort. When week 4 taxonomic data was coupled with PICRUS-based predicted metabolic functional data, differences in the functional potential of the microbiota, with propionate metabolism and fatty acid biosynthesis were predicted to be significantly higher in the RW+ cohort at week 4, but not week 8. Thus, initial changes to the gut microbiota observed after four weeks of voluntary exercise preceded changes in host expression of genes involved in energy intake. This study suggests a time dependent, link between physical exercise, the gut microbiome and energy balance.



## 4.2 INTRODUCTION

Physical exercise has been shown to reduce systemic inflammation and is an excellent therapeutic intervention for obesity and type-2-diabetes (T2D), with effects often shown to be as beneficial (in terms of efficacy) as pharmaceutical interventions (Booth et al. 2002, Group 2002, Gleeson 2007). Using the diet-induced obesity (DIO) mouse model, it has been shown that physical exercise reverses obesity associated hyperglycaemia, body fat, body weight, and alters the gut microbiota (see below) as well as markers of inflammation (Bradley et al. 2008, Huang et al. 2010). Furthermore, exercise has been associated with the improvement of several non-obesity related conditions such as osteoporosis and lowering the risk of developing several GI diseases such as colon cancer and inflammatory bowel diseases (IBDs) (Booth et al. 2002, Martin 2011, Sanchez et al. 2012, Khalili et al. 2013). Thus, there has been a growing interest in understanding the related mechanisms associated with physical exercise.

Similarly, there have been ever greater efforts to determine if the gut microbiota plays a role in exercise-induced alterations in the balance between energy intake and expenditure that determines body weight, as this knowledge could be used to optimise the effects of exercise and in the general population and provide better health outcomes for patients with a wide range of conditions including obesity and associated T2D and inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (UC) (Booth et al. 2002, Group 2002, Gleeson 2007, Dicksved et al. 2008, Machiels et al. 2013). Notably, it is increasingly appreciated that humans may now be thought of as "superorganisms" owing to the metabolic potential not only encoded by our own genomes but also that encoded within our associated microbial metagenome. It has been estimated that the human microbiota contains

more than 1000 bacterial species each possessing a genome encoding an average of 5000 genes resulting in a microbial genetic repertoire far larger than that of the human host (Xu et al. 2003, Eckburg et al. 2005) and therefore capable of performing metabolic functions not encoded by the host genome. The vast majority of these microbial inhabitants (~1.5kg or 100 trillion cells) reside within the gut leading to the gut microbiota being heralded as the body's "forgotten organ" (O'Hara and Shanahan 2006, Consortium 2012). These bacterial gut residents have been shown to play a key role in dietary energy harvest through the action of microbial enzymes. Bäckhed *et al.* showed germ-free mice develop less body fat than their conventionally raised counterparts, however when germ-free mice were conventionalized, a dramatic increase in body fat was observed in spite of decreased energy intake (Bäckhed et al. 2004).

While several studies have examined the effect of obesity and high fat feeding on the composition of the gut microbiota, to date relatively few studies have examined the impact of exercise on the gut microbiota and, in turn, energy balance. In a recent study by Clarke *et al.*, the bacterial diversity of professional athletes was shown to be significantly higher than that of both high and low BMI controls (Karlsson et al. 2012, Everard et al. 2013, Clarke et al. 2014). With respect to murine studies, Evans *et al.* assessed how a diet consisting of 60% energy (high) fat, 20% energy protein and 20% energy carbohydrate (high fat diet; HFD) affects the gut microbiota in mice that had access to running wheels (RW+) for 14 weeks. Voluntary exercise was shown to reduce weight gain and alter the composition of the gut microbiota compared to sedentary (RW-) controls. By feeding a 10% energy (low) fat, 20% energy protein and 70% energy carbohydrate enriched diet (LFD) to RW+ mice, the same study demonstrated that physical exercise does not significantly change body

weight but still altered the composition of gut microbial profiles at week 12 along with food intake at week 13, albeit to a lesser degree than observed in the HFD fed animals (Evans et al. 2014).

While these studies provide an insight into the effect of exercise on gut microbial composition, variation in (1) diet, (2) species differences and (3) alteration in body weight make drawing direct conclusions between exercise, gut microbiota composition and energy balance difficult. Using the previously described LFD fed C57BL/6 RW+ mouse model (Evans et al. 2014) here we gained an insight into a time dependent effect linking physical activity, gut microbiota, and energy balance, without the confounding effects of high fat intake or exercise induced alteration in body weight.

## **4.3 MATERIALS AND METHODS**

### **4.3.1 Ethics**

All research procedures involving mice were licenced under the Cruelty to Animals Act 1976 and ethical approval was obtained via the University College Cork (UCC) Animal Ethical Review Committee (#2011/005).

### **4.3.2 Animals**

Three week old male C57BL/6J mice (Harlan, Oxon, UK) were individually caged upon arrival and acclimatised for 2 weeks in individually ventilated cages. Animals were housed in the following controlled environment: light (06.00-18.00), temperature ( $21\pm1^{\circ}\text{C}$ ) and humidity (45-65%) and allowed free access to drinking water and a LFD (10% kJ fat and 20% kJ casein; #D12450, Research diets, New Brunswick, NJ, USA).

### **4.3.3 Experimental protocol**

Following the 2 week acclimatisation period, weight matched mice were housed individually with (RW+) or without (RW-) access to running wheels (n=8/group)(Figure 1). Mice were given the LFD with free access to drinking water and food. Body weights and food consumption were measured weekly with the exception of week 5 where food consumption could not be measured due to technical issues. During weeks 6-7, both food intake and metabolic activity were measured during light and dark phases using TSE PhenoMaster cages (TSE systems, Bad Homburg, Germany)(Figure 1). Fecal pellets were taken from all cages at weeks 0, 4 and 8 to allow for the examination of gut microbial population *via* Illumina MiSeq DNA sequencing. At the end of the trial period, animals were fasted for 6 hours and their body composition analysed using the Bruker minispec LF50H

(Bruker optics, Ettlingen, Germany). Mice were then anaesthetised with a co-administration of Ketamine (65 mg/kg bodyweight) and Xylazine (13 mg/kg bodyweight). Blood was taken from anaesthetised mice into EDTA treated tubes (BD, USA) and treated with both Aprotinin and Diprotin A at a final concentration of 500,000 KIU/L and 0.1mM respectively (Sigma, Ireland) to protect from proteolytic digestion. Plasma was separated from blood by centrifugation at 2000rpm at 4°C for 15 minutes. Mice were then sacrificed by cervical dislocation and tissues of interest dissected, weighted and either snap frozen in liquid nitrogen (subcutaneous, epididymal, stomach, intestine, colon, cecum, liver) or on dry ice (brain). Plasma and tissues were then stored at -80°C prior to analysis.

#### **4.3.4 Analysis of metabolic activity**

The experimental protocol described by McAllan *et al.* (McAllan et al. 2013, McAllan et al. 2014) was used to analyse food intake, metabolic activity ( $\text{VO}_2$ ; oxygen consumption) at 9 minute intervals over the study period, with the exception that running wheels (used in the home cages) were introduced into the TSE PhenoMaster cages upon housing the RW+ mice. Mice had *ad libitum* access to LFD and water during the ~3 day housing period in the PhenoMaster cages. After the initial 17h acclimatisation period, wheel rotations were automatically recorded on day 1 (Figure 1). Although the RW+ mice had access to RW in day 2, the RW sensor was disconnected on this day in order to measure metabolic activity. Food intake was measured over day 1 and 2, averaged. Respiratory exchanged ration (RER) was determined using  $\text{VCO}_2$  and  $\text{VO}_2$  data recorded on day 2.

#### **4.3.5 Microbial DNA extraction, 16s rRNA amplification and Illumina MiSeq sequencing**

Fecal pellets were collected from each animal on the trial at weeks 1, 4 and 8. Total metagenomic DNA was extracted from these fresh pellets with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) where, an additional bead beating step was incorporated into the protocol (Yu and Morrison 2004). Extracted DNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Ireland). The V3-V4 variable region of the 16s rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina). Initially the template DNA was amplified using primers specific to the V3-V4 region of the 16s rRNA gene which also allowed for the Illumina overhang adaptor, where, the forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse primer (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used. Each PCR reaction contained 2.5µl DNA template, 5 µl forward primer (1µM), 5 µl reverse primer (1µM) (Sigma, Ireland) and 12.5 µl Kapa HiFi Hotstart Readmix (2X) (KAPA Biosystems Inc, USA). The template DNA was amplified under the following PCR conditions for a total of 25 cycles: 95°C for 3 minutes and 30 seconds respectively (initialization and denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (elongation) followed by a final elongation period of 5 minutes. A negative control reaction whereby the template DNA was replaced with PCR grade water was employed to confirm lack of contamination and PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose gel, 100v) post PCR reaction.

Successful amplicons were then cleaned using the AMPure XP purification system (Beckman and Coulter, Takeley, UK). A second PCR reaction was then completed using the previously amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample to allow all samples to be pooled, sequenced on one flow cell and subsequently identified bioinformatically. Each reaction contained 25 µl Kapa HiFi HotStart ReadyMix (2X), 5 µl template DNA, 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx) and 10 µl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just 8 cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) in conjunction with the broad range DNA quantification assay kit (Life technologies, Carlsbad, CA, USA)). All samples were then pooled to an eqimolar concentration and the pool underwent a final cleaning step. Quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2M NaOH, diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX. Samples were then sequenced in house on the MiSeq sequencing platform at the Teagasc Next Generation sequencing platform centre (Teagasc Food Research Centre, Moorepark) using a 2x300 cycle V3 Kit following protocols outlined by Illumina (Fouhy et al. 2015).

#### **4.3.6 Bioinformatic analysis**

Three hundred base pair paired-end reads were assembled using FLASH (fast length adjustment of short reads to improve genome assemblies). Paired-end reads were further processed with the inclusion of quality filtering based on a quality score of > 25 followed by subsequent removal of mismatched barcodes and sequences below

length threshold using QIIME. USEARCH v7 (64-bit) was then used for denoising and chimera detection as well as clustering into operational taxonomic units (OTUs). PyNAST was used to align OTUs and taxonomy was assigned by using BLAST against the SILVA SSURef database release 111. QIIME was used to generate Alpha and Beta diversities, calculated based on Bray Curtis, Weighted and UnWeighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were then visualised using EMPEROR v0.9.3-dev. Predictive functional profiling was performed using the PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states) tool (Langille et al. 2013).

#### **4.3.7 RNA extraction and Real-Time (RT) PCR analysis**

Total RNA was extracted from hypothalamic blocks using the RNeasy lipid mini kit (Qiagen) according to manufacturer's protocols. Potential DNA contamination was eliminated by the incorporation of DNase (Qiagen) treatment during the RNA extraction process. Reverse transcription of 1 µg of RNA was achieved using 2.5 ng/µl random hexamer primers (Bioline, London, UK), 2 U/µl RNase inhibitor (Promega, Madison, USA), 0.5 mM dNTP (Promega) and the Superscript II first stand system (Invitrogen) in accordance with manufacturer's instructions. Gene expression was measured using the Roche Lightcycler 480 system (Roche, Rotkreuz, Switzerland) by the amplification of 1 µl complementary DNA using the Lightcycler SYBR Green I Mastermix kit (Roche, Penzberg, Germany) with 2.5 µM gene specific primers (Table 1). Complimentary DNA was amplified under the following PCR conditions for a total of 50 cycles: 95°C for 10 minutes (initial denaturation), 95°C for 10 seconds (denaturation), 58-65 °C for 5 seconds (annealing) and 72°C for 5 seconds (elongation). The authenticity of PCR products was determined by melting curve analysis and using automated sequencing. Data corresponding to



crossing point (Cp) values were normalised to the expression of the housekeeping genes  $\beta$ -actin. ( $\Delta\Delta C_p = \Delta C_p \text{ target gene} - \Delta C_p \text{ housekeeping gene}$ ).

#### **4.3.8 Plasma analysis**

ELISA were used to quantify plasma levels of Insulin (Crystal Chem, Downers Grove, IL, USA), glucagon-like peptide 1 (GLP-1) (Millipore, St. Charles, MO, USA), Peptide YY (PYY) (Phoenix Pharmaceuticals), Ghrelin (EMD Millipore), and glucose (Calbiochem, Darmstadt, Germany).

#### **4.3.9 Statistical analysis**

Data were analysed by Two-Sample T-Test. ANCOVA was used to assess the effect of RW on  $VO_2$ , with body weight as the co-variate. Non-parametric Kruskal-Wallis analysis was carried out to determine statically significant taxonomic differences between the RW+ and RW- groups where statistical significance was accepted as  $p < 0.05$ . Spearman correlations were used to examine relationships between the measured parameters where again significance was accepted as  $p < 0.05$ . Statistics were performed using Graphpad prism (ver. 6.04; San Diego, CA, USA) and Minitab statistical software package (ver. 15; State College, PA, USA)

## 4.4 RESULTS

### 4.4.1 Voluntary exercise altered energy balance.

Body weights of the RW+ mice did not significantly differ from RW- mice at any point during the 8 week trial period (Figure 2A), which was reflected by unchanged % fat and lean mass (Figure 2C) and by tissues weights (Table 2). In contrast, in the home cages, voluntary exercise increased food intake, becoming significant at weeks 3 and 4 in the RW+ compared to the RW- control group ( $P<0.05$ )(Figure 2B), and in weeks 6 and 7 when the feeding behaviour was further characterised using TSE Phenomaster cages. The metabolic cage analysis further revealed that mice in RW+ group had significantly increased dark phase food consumption compared to RW- counterparts ( $p<0.05$ ) (Figure 3A) along with significantly increased use of the running wheels during dark phase compared to light phase, as measured by running wheel rotations (left and right)( $p<0.05$ ) (Figure 3B). Energy expenditure, as determined by  $VO_2$ , was significantly increased in the RW+ compared to RW- ( $p<0.05$ ) during both light and dark phases (Figure 3C).

To understand the mechanisms underlying the increased food intake in RW+ group, plasma levels of several gastrointestinal derived hormones and associated hypothalamic neuropeptide gene expression were examined. Plasma levels of GLP-1, PYY and ghrelin showed no significant difference between the two groups. Hypothalamic expression of orexigenic neuropeptide Y (NPY) and anorexigenic pro-opiomelanocortin (POMC) was unaltered in RW+ group while gene expression of fatty acid synthase (FASN) and peroxisome proliferator activated receptor alpha ( $PPAR\alpha$ ) both showed a notable trend towards an increase in the RW+ cohort compared to the RW- cohort ( $P=0.071$  and  $0.052$ , respectively) (Figure 4).

#### **4.4.2 Voluntary exercise and carbohydrate metabolism**

The RER calculated based on metabolic data generated at week 6 and 7 using TSE PhenoMaster cages revealed an increased metabolism of carbohydrate (RER close to 1) in the dark phase in the RW+ group compared to RW- (Figure 3D) and yet plasma insulin and glucose of both groups were not significantly different. This was accompanied by a notable trend towards significantly higher expression of genes involved in cellular glucose uptake in the hypothalamus, namely insulin receptor (INSR) ( $p=0.085$ ), glucose transporter 1 (Glut1) ( $p=0.085$ ) and insulin receptor substrate 1 (IRS1) ( $p=0.065$ )(Figure 4).

#### **4.4.3 Voluntary exercise does not alter microbial diversity and invokes only minor changes to gut microbial taxonomies.**

A total of 11,513,829 V3-V4 16s rRNA paired end sequence reads were generated during the sequencing process, corresponding to an average of 239,871 reads per animal per time point. Alpha diversity values were calculated (Figure 5) for species richness (Chao1), biodiversity (Shannon index), observed species and species number relative to abundance within the sample (Simpson diversity index). At week 0 (i.e. before any exercise took place), values for both Chao1 and observed species were statistically higher in the RW+ cohort compared to the RW- cohort, a trend which continued at weeks 4 and 8. No other significant differences were observed in  $\alpha$ -diversity. Principal coordinate analysis (built upon the unweighted unifracs algorithm) (Figure 6) did not reveal any distinct clustering between the two groups within the trial at any of the examined time points.

Phylogenetic analysis (examined at phylum, family and genus level) detected minor changes in microbial taxonomy between RW+ and RW- animals (Table 3). At week

0, significantly higher relative proportions of the families *Veillonellaceae* and *Moraxellaceae* ( $p=0.046$  and  $0.002$ , respectively) and the genera *Acidominococcus* and *Morella* ( $p=0.027$  and  $0.002$ , respectively) were detected in the animals that were subsequently assigned as the RW+ cohort compared to the RW- animals. By week 4 of the experiment, the RW+ cohort showed significantly lower relative abundances of Cyanobacteria ( $p=0.016$ ) at phylum level, significantly higher proportions of *Bacteroidaceae* and *Rhodospirillaceae* ( $p=0.046$  and  $0.046$ , respectively) at family level and *Bacteroides* and *Mogibacterium* ( $p=0.046$  and  $0.043$ , respectively) at genus level compared to the RW- group. Conversely, the RW- sequence data showed significantly higher proportions of *Clostridaceae* ( $p=0.035$ ) at family level and *Clostridium* and *Blautia* ( $p=0.019$  and  $0.028$ , respectively) at genus level when compared to that of the RW+ animals. At the trial's end point (week 8), exercising animals showed significantly lower populations within the Cyanobacteria ( $p=0.002$ ) phylum but significantly higher proportions within the *Coriobacteriaceae* and *Alcaligenaceae* ( $p=0.016$  and  $0.025$ , respectively) families and the *Enterorhabdus* and *Parasutterella* ( $p=0.036$  and  $0.025$ , respectively) genera when compared to those populations within the RW- group.

#### **4.4.4 Voluntary exercise-induced food intake positively correlates with gut microbial diversity.**

Correlations between metabolic parameters and both microbiota composition and diversity were examined using Spearman correlations. In the RW+ cohort, significant negative correlations were observed between *Deferribacteres* (phylum), *Deferribacteraceae* (family), *Mucispirillum* (genus) ( $p=0.007$ ,  $r=-0.929$ ) and *Coprococcus* (genus) ( $p=0.024$ ,  $r=-0.857$ ) and voluntary exercise while conversely,

*Clostridium* proportions were significantly positively correlated with exercise ( $p=0.048$ ,  $r=0.802$ ). Spearman correlations also revealed a significant positive correlation between  $VO_2$  and the taxonomic family *Rikenellaceae* while a strong positive correlation was observed between total food consumed and the Simpson and Shannon alpha diversity indices ( $p=0.034$ ,  $r=0.821$ ).

#### **4.4.5 PICRUSt analysis revealed alterations to metabolic pathways due to voluntary exercise.**

A PICRUSt analysis was carried out on both week 4 and week 8 sequence data. PICRUSt analysis of week 4 sequences predicted significantly altered metabolic pathways between the gut bacteria of the RW+ and RW- cohorts, where, carbohydrate metabolism, specifically propanoate metabolism ( $p=0.036$ ) and lipid metabolism, specifically fatty acid biosynthesis ( $p=0.027$ ) were significantly higher in the RW+ cohort. In contrast, PICRUSt analysis of week 8 data did not suggest any significant differences between the two groups in terms of carbohydrate metabolism or fatty acid biosynthesis pathways.

## 4.5 DISCUSSION

This study aimed to determine the effect of voluntary exercise on host metabolic parameters and the composition and diversity of the gut microbiota in C57BL/6 mice fed a LFD. Results from this study suggest that voluntary exercise alters energy balance and invokes subtle changes to gut microbial communities in a time dependent manner.

Voluntary exercise has been shown to increase food intake in rodents (Garland et al. 2011, Evans et al. 2014). We extend these findings by showing that degree of exercise dictates changes in energy balance, as mice in the RW+ group did not change food intake in weeks 1 and 2, but increased consumption only after 3 weeks of running wheel activity. Mice in the RW+ group exercise more in the dark phase and consumed more food compared to RW- controls. The increased food intake was not related to plasma ghrelin, PYY or GLP-1 or associated hypothalamic POMC and NPY gene expression, suggesting an alternative mechanism by which RW activity increased food intake in the animals. Notably, both hypothalamic FASN and PPAR $\alpha$  expression have been shown to play important roles in food intake, as inhibition of FASN resulted in dramatic weight loss in BALB/c mice which was attributed to the inhibition of feeding. Moreover, mice with pancreatic and hypothalamic inactivated FASN show reduced food intake, which was normalised upon administration of a PPAR $\alpha$  antagonist into the hypothalamus (Chakravarthy et al. 2007). These data indicate an important role for hypothalamic FASN/PPAR $\alpha$  pathway in the control of food intake. It remains to be determined if this hypothalamic pathway plays a role in exercise induced food intake, given that in the current study, their gene expression showed a trend towards an increase in the RW+ group compared with that of the RW- group. It is also noteworthy that metabolic

activity (as determined by VO<sub>2</sub> measurements) was highest in the dark phase in RW+ compared to RW- (either phase), as was food consumption, when running wheel rotations were highest. This further supports the above suggestion that degree of voluntary exercise impacts on energy intake and expenditure. In the current study, the LFD consisted mainly of carbohydrate (70% energy). This along with the increased food intake recorded during the dark phase in the RW+ group in comparison to the control group, are consistent with the high RER value ( $1.04 \pm 0.01$ ) obtained in the RW+ group in the dark phase, indicating carbohydrate metabolism. Notably RER values exceeding 1.0 have previously been associated with intense physical activity (Jondeau et al. 1992). However, plasma insulin and glucose levels were not significantly different between groups.

With respect to the mouse microbiota, alpha diversity differences originated from before separation of the RW+ and RW- groups and did not change significantly thereafter. These differences likely arose from either or litter or cage variations, which have been shown to have the ability to invoke considerable changes to the murine gut microbiota in terms of both composition and diversity (Whittaker 1972, Benson et al. 2010, Hildebrand et al. 2013). Beta diversity measured by the unweighted unifracs distances matrix (Figure 6) showed no clear separation between the two groups. This close clustering is consistent with results reported by Allen *et al.* in a recent study, who reported a similarly proximal clustering between exercising RW+ and RW- C57BL/6 mice both consuming a LFD after a 6 week trial period. Another comparable study conducted by Evans *et al.* showed a larger separation between control and voluntarily exercising mice after a 12 week period, and thus exercise may invoke more substantial changes to gut microbial profiles if sustained over a longer period of time (Evans et al. 2014, Allen et al. 2015).

The aforementioned increased energy intake and nutrient uptake observed within the RW+ cohort may, in some part, be modulated by the gut microbiota. High throughput DNA sequencing of fecal pellets taken at week 4 showed significantly higher proportions of the *Bacteroidetes* genus in the RW+ group compared to the RW- group. Studies have shown that the *Bacteroides* genus harbour a wide amount of carbohydrate utilisation enzymes and therefore considerable saccharolytic potential is associated within this genus (Salyers et al. 1977, Salyers et al. 1977, Xu et al. 2003). Bäckhed *et al.* showed a 23% increase in body total body fat in germ free mice when colonised with *Bacteroides thetaiotaomicron*. This increased total body fat was accompanied by increased hepatic expression of FASN to facilitate the uptake of monosaccharaides (produced by the processing of dietary polysaccharides by microbial glycosylhydrolases) and their subsequent conversion in the liver to fatty acids (Bäckhed et al. 2004). Hooper *et al.* showed that *B. thetaiotaomicron* modulates the expression of several important intestinal functions including glucose uptake (Hooper et al. 2001). In this context it is interesting to note our hypothalamus-related observation that expression of FASN trended towards an increase, and was accompanied by similar changes in the insulin signalling pathway involved in glucose uptake, specifically a trend towards an increased expression of INSR, Glut1 and IRS1 compared to RW-. Interestingly, PICRUST analysis of week 4 sequence data predicted higher numbers of metabolic pathways for fatty acid biosynthesis among the RW+ group.

As well as potentially playing a role in increased expression of FASN and glucose uptake, exercise may also drive changes in the gut microbiota that improve gut health by the production of short chain fatty acids (SCFAs). SCFAs are produced by the microbial fermentation of dietary macronutrients (primarily carbohydrates). The



major SCFAs found within the gut ecosystem are acetate, propionate and butyrate and are thought to influence host health by a variety of different mechanisms which include, providing energy for host tissues, exerting anti-inflammatory effects and maintaining microbial balance within the gut and members of the *Bacteroidetes* genus have been shown to produce these SCFAs indeed, fecal propionate levels have been significantly correlated to their relative abundance (Cummings 1981, Topping and Clifton 2001, Salonen et al. 2014, Flint et al. 2015). PICRUST analysis further supported the production of SCFAs where it predicted the RW+ cohort had significantly higher propionate production metabolic pathways resulting in the formation of propionate. Conversely, significantly lower proportions of the SCFA producing genus *Blautia* were observed within the RW+ cohort at the same time point.

Taxonomic analysis of sequence data generated at week 8 showed significant increases in proportions of *Coriobacteriaceae* and *Alcaligenaceae* at family level which corresponded to *Enterorhabdus* and *Parasutterella*, respectively, at genus level, in RW+ animals. Notably, both of these genera have been associated with the microbiota of lean mice compared with their obese and diabetic counterparts. A 2011 study by Geurts *et al.* showed *Enterorhabdus* to be present within the gut microbiota of lean mice but was not detected in the microbiota of either obese or diabetic animals. The authors suggested a relationship between specific gut bacteria and the regulation of certain important physiological systems such as the endocannabinoid (eCB) and apelin systems which play important roles in several physiological functions. This significant increase in the proportions of *Enterorhabdus* observed within the RW+ group may provide a mechanism through which sustained voluntary exercise can invoke microbially-mediated regulation of

important host genes. Zhang *et al.* showed a significant increase in *Parasuterella* upon switching DIO C57BL/6 mice to a normal chow diet after 12 weeks of high fat diet feeding, a switch which restored overall structure, composition and bacterial diversity (Geurts et al. 2011, Zhang et al. 2012).

Finally, Spearman correlation analysis highlighted some potential links between voluntary exercise, energy intake and microbial taxonomies. A negative correlation was observed between voluntary exercise and *Coprococcus*. This is unexpected as *Coprococcus* has come to be regarded as a desirable component of the microbiota, as a producer of SCFAs and genus that is present in higher populations in elderly populations living in the community relative to those in long time care (Claesson et al. 2012, Louis et al. 2014, Reichardt et al. 2014). The strong significant negative correlation between running activity and *Mucispirillum* was noteworthy given the previous association with this genus and ulcerative colitis (Robertson et al. 2005, Cook et al. 2013). Strong positive correlations were also observed between total food consumed and both the Shannon and Simpson diversity indices. Given the significant increase in food consumption observed within the RW+ cohort, this correlation suggests that over a longer trial period, exercise driven increased food consumption may lead to increased bacterial diversity, a key marker of gut health (Shanahan 2003, Shanahan 2003, Moran and Shanahan 2014). It is important, however, to remember that Spearman correlations are a direct parameter to parameter numerical comparison and do not take into account the complex interplay which exists within the gut microbiota. Therefore while these correlations suggest links to potential roles which parameters such as running wheel rotations may have on particular taxonomic populations, conclusions relating to the effect which voluntary exercise invokes on the gut microbiota are better drawn from the

differences observed between the RW+ and RW- groups (as determined by the Kruskal-Wallis test).

In summary, the present data demonstrates that voluntary exercise increases energy intake, metabolic activity, and carbohydrate utilisation and are associated with subtle changes in the gut microbiota in C57BL/6 mice, with initial changes to the gut microbiota (observed at week 4) potentially contributing to subsequent changes in hypothalamic gene expression and associated increased energy intake.

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**Table 1.** Primers used for RT-PCR to determine hypothalamic gene expression.

	Forward primer (5'-3')	Reverse primer (5'-3')
POMC	5'-gggcaagcgctcctactccatg-3'	5'-ctcgccttccagctccctcttg-3'
NPY	5'-ccctcgctctatctctgctcgtgtg-3'	5'-gtagtatctggccatgtcctctgc-5'
IRS-1	5'-gcgcaaggcaccatctcaacaacc-3'	5'-gcacgcacccggaagggaacc-3'
PPAR $\alpha$	5'-atgggggtgatcggaggctaatag-3'	5'-gggtggcagggaagggaacagac-3'
GLUT1	5'ggcctgcccgaagatc-3'	5'-aggagctggagcaaaggac-3'
FASN	5'-tccacctttaagttgcctg-3'	5'-tctgctctcgtcatgtcacc-3'
IR	5'-gatttcccaacgtgtcctctac-3'	5'-caatgcggtagccagtggaagtg-3'
$\beta$ -actin	5'-agagggaatcgtgcgtgac-3'	5'-caatagtgatgacctggcgt-3'

RT\_PCR primer sequences for examination of the hypothalamic expression of POMC, Pro-opiomelanocortin; NPY, Neuropeptide Y; IRS-1, Insulin receptor substrate 1; PPAR $\alpha$ , Peroxisome proliferator activated receptor alpha; FASN, Fatty acid synthase; IR, Insulin resistance and  $\beta$ -actin.

**Table 2.** Harvested tissues/organ weights.

<b>Tissue/Organ (g)</b>	<b>RW-</b>	<b>RW+</b>
<b>Sub Cutaneous (as % of BW)</b>	1.49 ± 0.13	1.35 ± 0.18
<b>Epididymal fat (as % of BW)</b>	2.03 ± 0.19	1.78 ± 0.16
<b>Stomach</b>	0.41 ± 0.06	0.43 ± 0.03
<b>Intestine</b>	0.91 ± 0.03	0.91 ± 0.03
<b>Colon</b>	0.23 ± 0.02	0.24 ± 0.03
<b>Cecum</b>	0.27 ± 0.01	0.28 ± 0.02
<b>Liver</b>	1.01 ± 0.04	0.93 ± 0.05

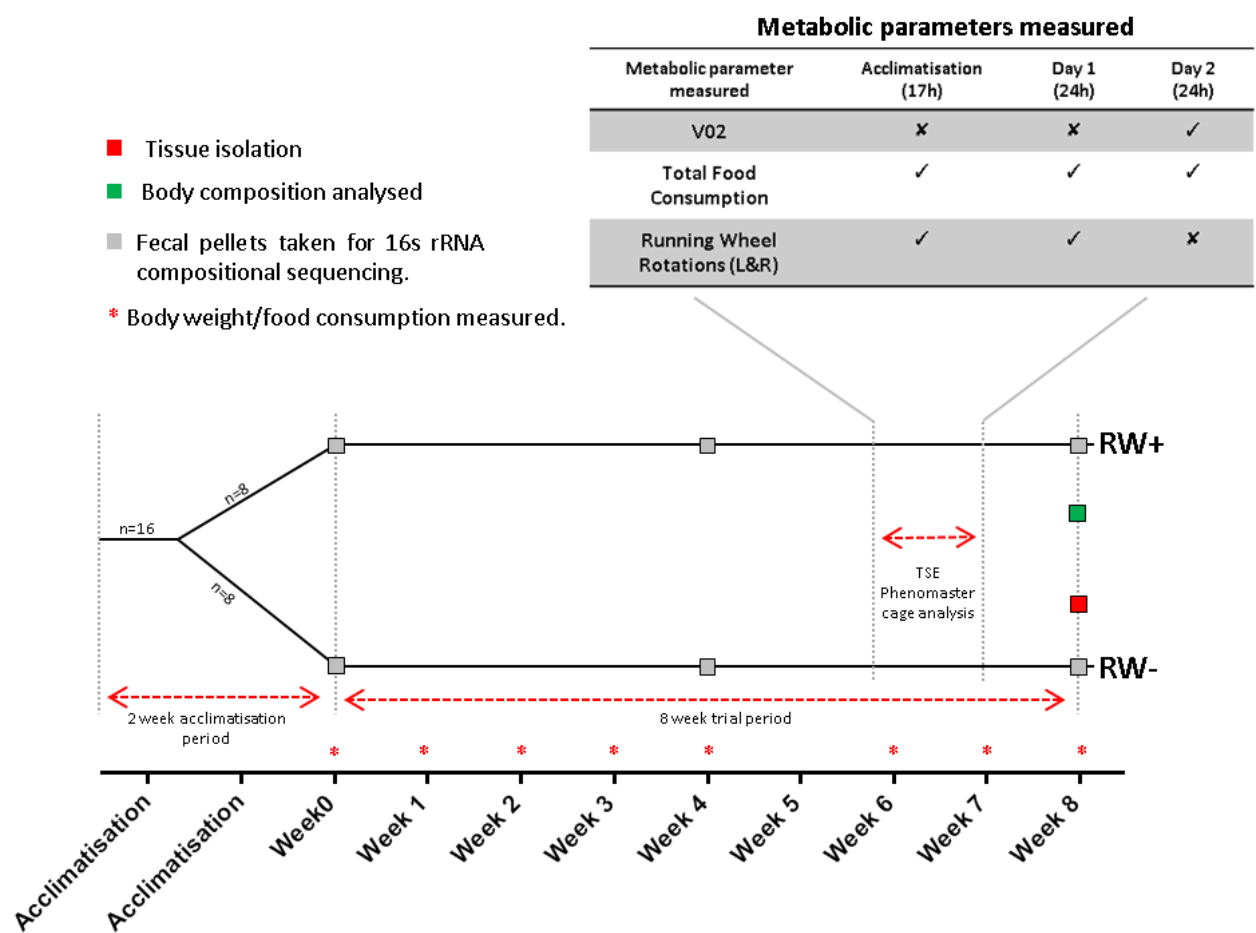
Animal tissues and organs weights (g) harvested post sacrifice. Data are means ± SEM.

**Table 3.** Significant taxonomic differences between RW + and RW – groups.

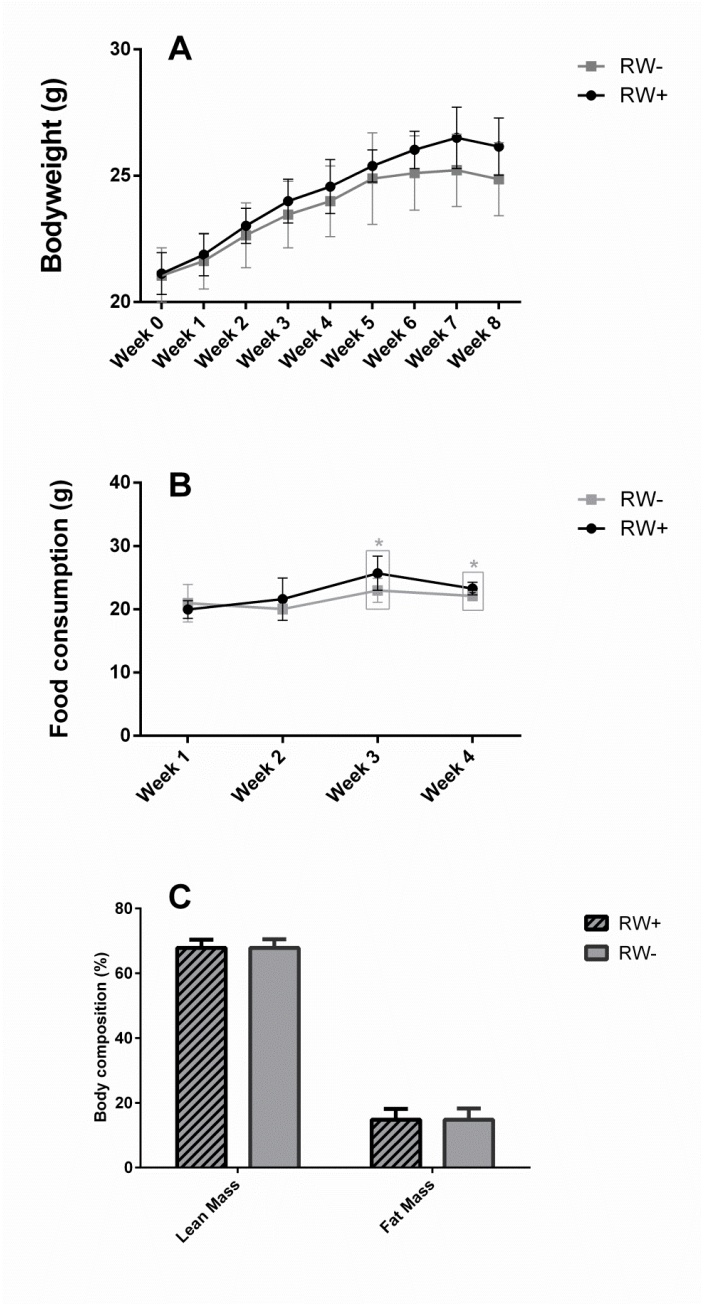
<b>Taxonomy</b>	<b>P-value</b>	<b>RW+ Group v RW- Group</b>
<b>Week 0</b>		
Group in which proportions are significantly higher		
<b>Family</b>		
Veillonellaceae	0.046	<b>RW+</b>
Moraxellaceae	0.002	<b>RW+</b>
<b>Genus</b>		
<i>Acidaminococcus</i>	0.027	<b>RW+</b>
<i>Moraxella</i>	0.002	<b>RW+</b>
<b>Week 4</b>		
<b>Phylum</b>		
Cyanobacteria	0.016	<b>RW-</b>
<b>Family</b>		
<i>Bacteroidaceae</i>	0.046	<b>RW+</b>
<i>Clostridiaceae</i>	0.035	<b>RW-</b>
<i>Rhodospirillaceae</i>	0.046	<b>RW+</b>
<b>Genus</b>		
<i>Bacteroides</i>	0.046	<b>RW+</b>
<i>Clostridium</i>	0.019	<b>RW-</b>
<i>Mogibacterium</i>	0.043	<b>RW+</b>
<i>Blautia</i>	0.028	<b>RW-</b>
<b>Week 8</b>		
<b>Phylum</b>		
Cyanobacteria	0.002	<b>RW-</b>
<b>Family</b>		
<i>Coriobacteriaceae</i>	0.016	<b>RW+</b>
<i>Alcaligenaceae</i>	0.025	<b>RW+</b>
<b>Genus</b>		
<i>Enterorhabdus</i>	0.036	<b>RW+</b>
<i>Parasutterella</i>	0.025	<b>RW+</b>

Statistically significant taxonomic differences between the RW+ and RW- groups observed at week 0, 4 and 8 where significance was taken at  $p < 0.05$  as determined by the Kruskal-Wallis non parametric test.

**Figure 1. Experimental overview.** Experimental overview showing timeline of the trial where; \* indicates weekly bodyweight/food consumption measurements, ■ indicates fecal pellet collection from individual mice for 16s rRNA compositional sequencing, ■ indicates point at which animal’s body composition was analysed using the Bruker minispec LF50H and ■ indicates point at which animal tissues were isolated (post sacrifice).

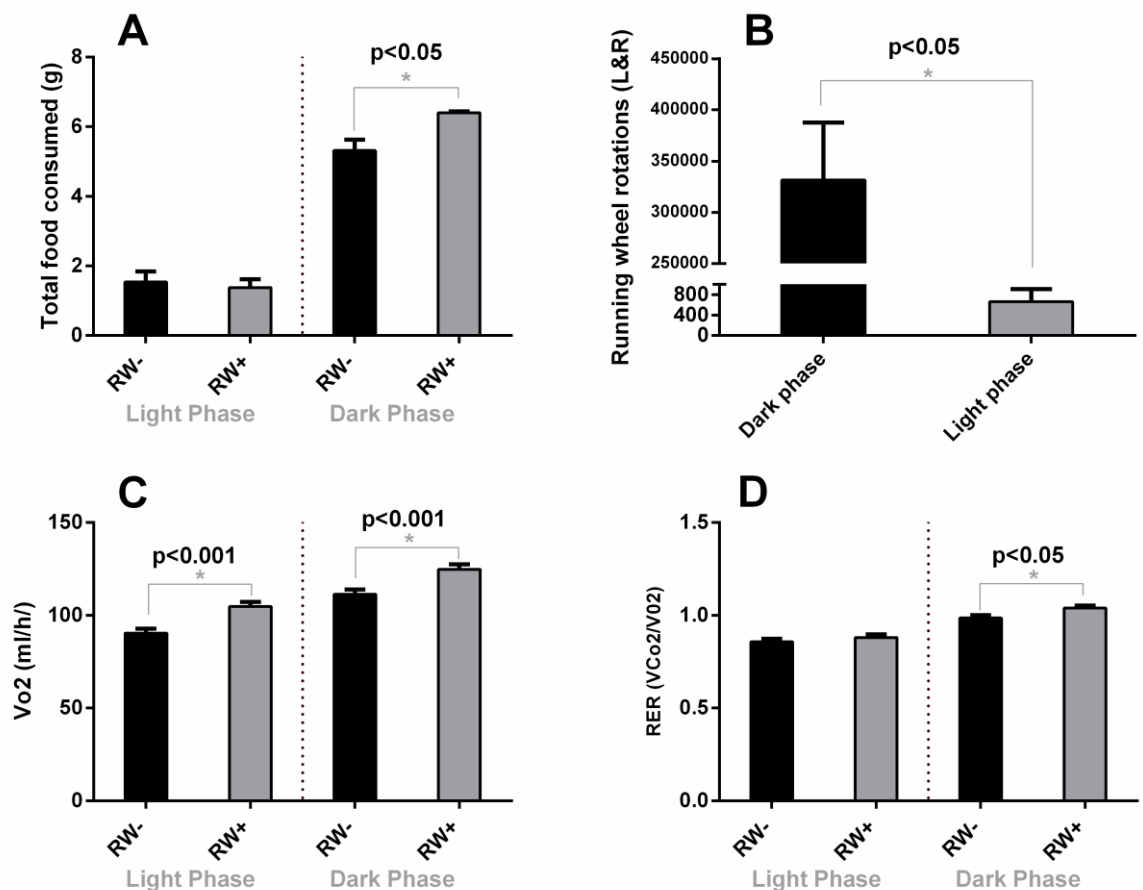


**Figure 2. Impact of voluntary exercise on body weight, food consumption and body composition.** (A) Body weight trajectories of mice during the 8 week experiment, (B) food consumption (up to week 5) and (C) body composition. Data represented are mean values  $\pm$  SEM and where \* determines significant differences between the two groups ( $p < 0.05$ ).

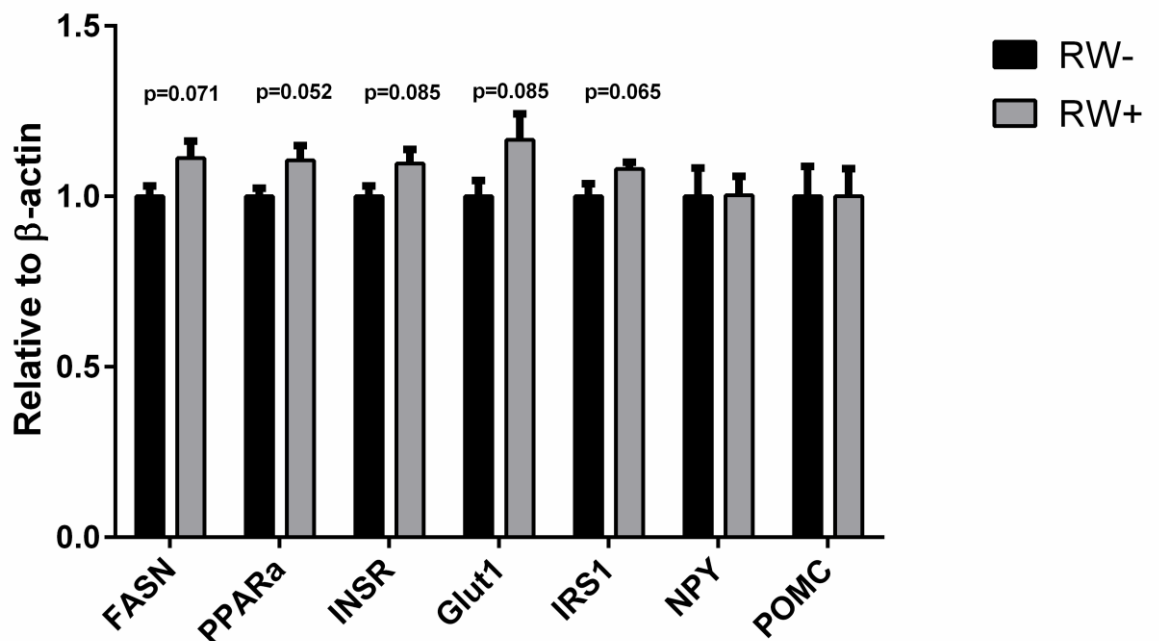




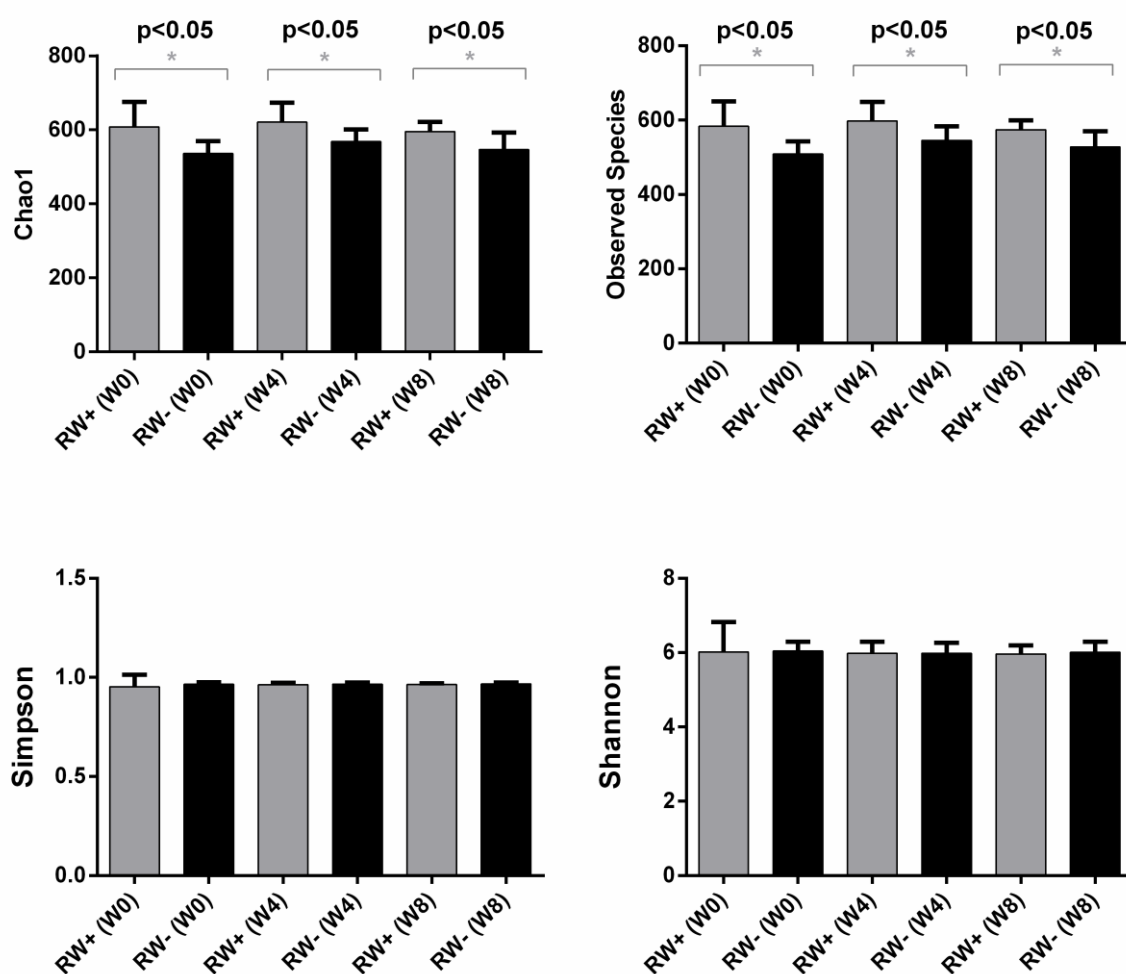
**Figure 3. Impact of voluntary exercise on metabolic activity.** Metabolic activity was measured using TSE Phenomaster cages at weeks 6-7 for mice with and without access to running wheels. Experimental data for; (A) total food consumption, (B) running wheel rotations (RW+ group), (C) oxygen consumption ( $\text{Vo}_2$ ) and (D) respiratory exchange ratio (RER) were collected from individual mice at 12 minute intervals during the 65 hour experimental period and are shown as mean values  $\pm$  SEM for light and dark phases. Statistical significance was determined using ANCOVA for  $\text{Vo}_2$  and a Two-sample T-Test for all other parameters where significance was taken as  $p < 0.05$ .



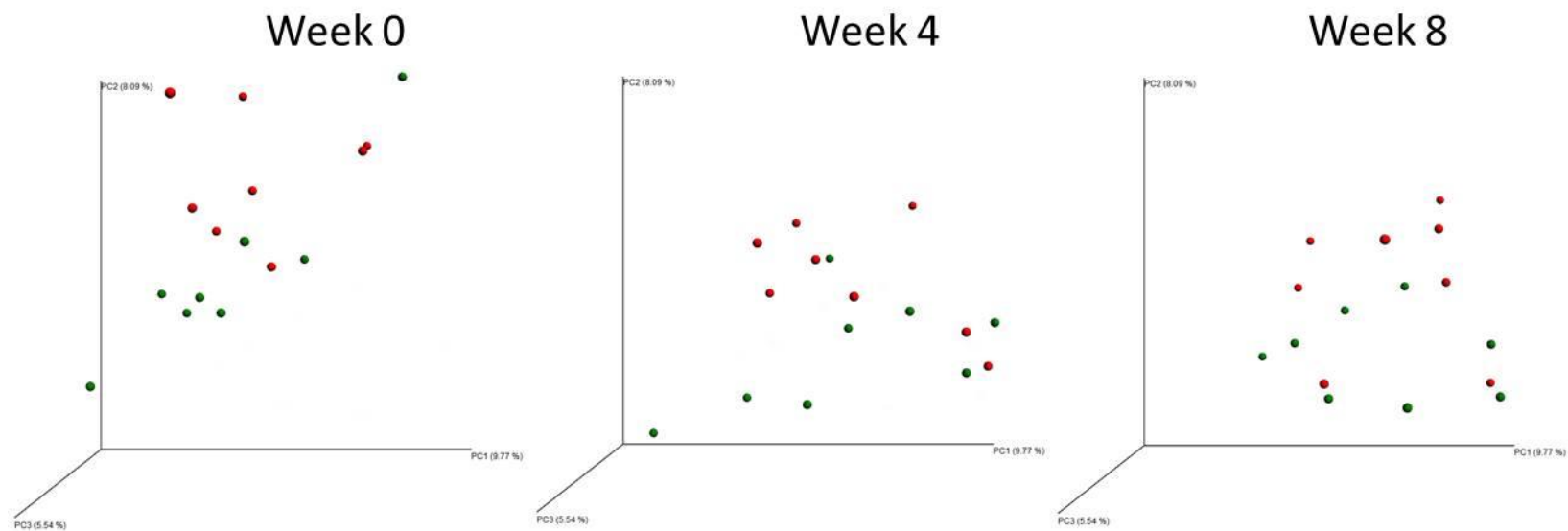
**Figure 4. Hypothalamic gene expression.** Hypothalamic gene expression was investigated in mice after 8 weeks of voluntary exercise on a standard low fat diet. Relative mRNA expression is shown here for fatty acid synthase (FASN), peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ), Insulin receptor (INSR), glucose transporter 1 (Glut1), insulin receptor substrate 1 (IRS-1), pro-opiomelanocortin (POMC) and neuropeptide (NPY). Data represent mean values  $\pm$ SEM (n=8 per group). Gene expression shown is relative to  $\beta$ -actin and statistical significance was determined where  $p < 0.05$ .



**Figure 5. Alpha diversity values.** Alpha diversity measurements of bacterial communities represented as means  $\pm$  SEM, where, Chao1 and observed species show significantly higher values within the RW+ group as determined using the Kruskal-Wallis algorithm ( $p < 0.05$ ).



**Figure 6. Impact of voluntary exercise on gut microbial composition.** Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of generated 16s rRNA sequences demonstrating no clear separation between the RW+ and RW- groups at weeks 0, 4 or 8, where, • points represent data generated from animals within the RW+ group and • represent points generated from animals within the RW- group.



## 4.7 SUPPORTING INFORMATION

**Table S1.** PICRUSt predicted metabolic pathway activity.

<i><b>Week 4 data- Higher in RW+ Cohort</b></i>	<i><b>P-Value</b></i>
Cellular.Processes.Cell.Growth.and.Death.Meiosis...yeast	0.093*
Cellular.Processes.Cell.Growth.and.Death.p53.signaling.pathway	0.093*
Environmental.Information.Processing.Signal.Transduction.Phosphatidylinositol.signaling.system	0.093*
Genetic.Information.Processing.Folding..Sorting.and.Degradation.Sulfur.relay.system	0.036**
Genetic.Information.Processing.Replication.and.Repair.Non.homologous.end.joining	0.093*
Genetic.Information.Processing.Transcription.Transcription.machinery	0.059*
Human.Diseases.Cancers.Colorectal.cancer	0.093*
Human.Diseases.Cancers.Small.cell.lung.cancer	0.093*
Human.Diseases.Cardiovascular.Diseases.Viral.myocarditis	0.093*
Human.Diseases.Immune.System.Diseases.Systemic.lupus.erythematosus	0.059*
Human.Diseases.Infectious.Diseases.Influenza.A	0.093*
Human.Diseases.Infectious.Diseases.Toxoplasmosis	0.093*
Metabolism.Amino.Acid.Metabolism.Phenylalanine.metabolism	0.036**
Metabolism.Amino.Acid.Metabolism.Tyrosine.metabolism	0.036**
Metabolism.Biosynthesis.of.Other.Secondary.Metabolites.Caffeine.metabolism	0.059*
Metabolism.Carbohydrate.Metabolism.Glyoxylate.and.dicarboxylate.metabolism	0.074*
Metabolism.Carbohydrate.Metabolism.Propanoate.metabolism	0.036**
Metabolism.Carbohydrate.Metabolism.Pyruvate.metabolism	0.074*
Metabolism.Energy.Metabolism.Sulfur.metabolism	0.059*
Metabolism.Glycan.Biosynthesis.and.Metabolism.Glycosyltransferases	0.046**
Metabolism.Lipid.Metabolism.Biosynthesis.of.unsaturated.fatty.acids	0.027**

**Table S1. (Continued)**

<b><i>Week 4 data- Higher in RW+ Cohort</i></b>	<b><i>P-Value</i></b>
Metabolism.Lipid.Metabolism.Fatty.acid.elongation.in.mitochondria	0.059*
Metabolism.Lipid.Metabolism.Linoleic.acid.metabolism	0.003**
Metabolism.Lipid.Metabolism.Steroid.biosynthesis	0.059*
Metabolism.Lipid.Metabolism.Steroid.hormone.biosynthesis	0.059*
Metabolism.Metabolism.of.Cofactors.and.Vitamins.Retinol.metabolism	0.027**
Metabolism.Metabolism.of.Terpenoids.and.Polyketides.Carotenoid.biosynthesis	0.059*
Metabolism.Metabolism.of.Terpenoids.and.Polyketides.Limonene.and.pinene.degradation	0.074*
Metabolism.Metabolism.of.Terpenoids.and.Polyketides.Tetracycline.biosynthesis	0.074*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Aminobenzoate.degradation	0.021**
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Atrazine.degradation	0.046**
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Bisphenol.degradation	0.009**
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Caprolactam.degradation	0.036**
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Chlorocyclohexane.and.chlorobenzene.degradation	0.074*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Drug.metabolism...cytochrome.P450	0.059*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Fluorobenzoate.degradation	0.059*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Metabolism.of.xenobiotics.by.cytochrome.P450	0.059*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Naphthalene.degradation	0.093*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Styrene.degradation	0.059*
Organismal.Systems.Circulatory.System.Cardiac.muscle.contraction	0.093*
Organismal.Systems.Endocrine.System.Insulin.signaling.pathway	0.046**
Organismal.Systems.Environmental.Adaptation.Circadian.rhythm...plant	0.059*

**Table S1. (Continued)**

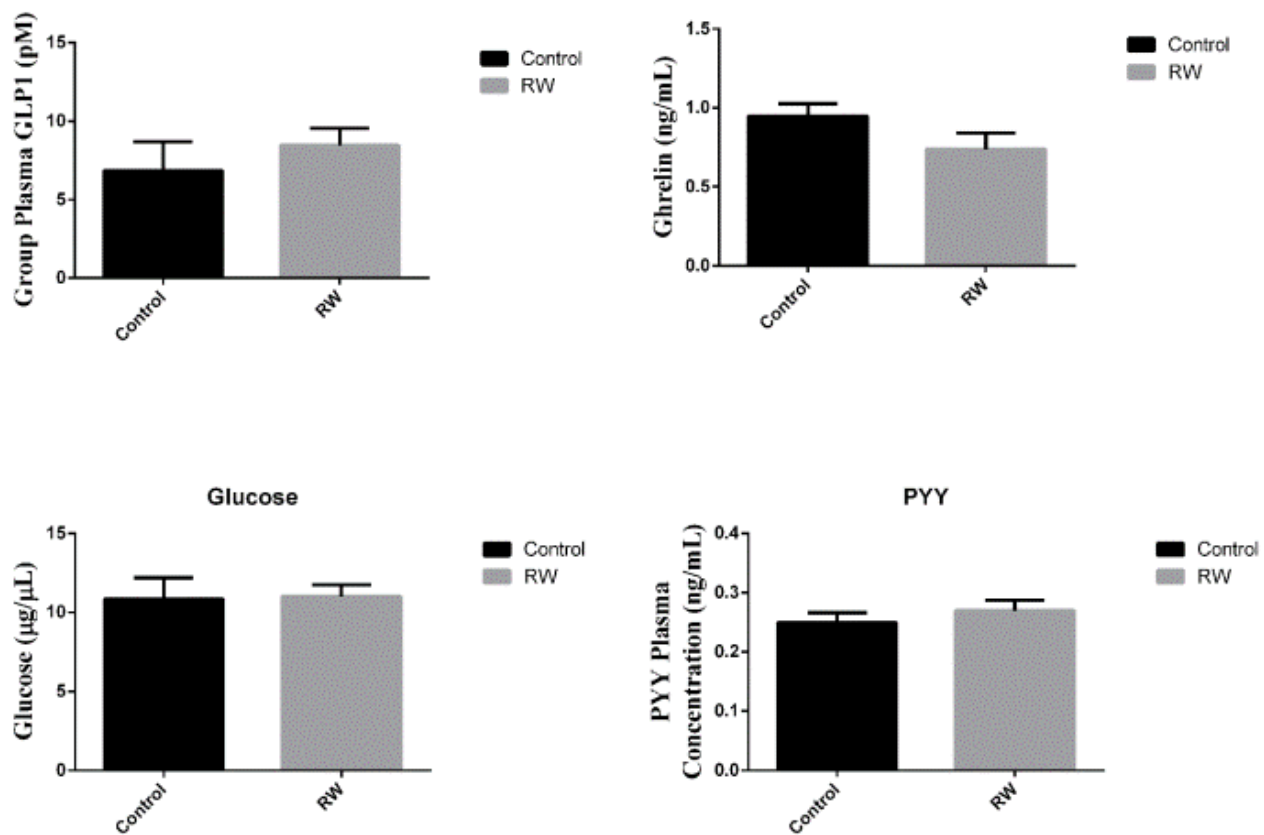
<b><i>Week 4 data- Higher in RW+ Cohort</i></b>	<b><i>P-Value</i></b>
Unclassified.Cellular.Processes.and.Signaling.Inorganic.ion.transport.and.metabolism	0.059*
Unclassified.Cellular.Processes.and.Signaling.Other.ion.coupled.transporters	0.009**
Unclassified.Cellular.Processes.and.Signaling.Signal.transduction.mechanisms	0.059*
Unclassified.Genetic.Information.Processing.Transcription.related.proteins	0.059*
Unclassified.Metabolism.Metabolism.of.cofactors.and.vitamins	0.046**
Unclassified.Metabolism.Others	0.046**
Unclassified.Poorly.Characterized.Function.unknown	0.046**
<b><i>Week 4 data- higher in RW- Cohort</i></b>	
Human.Diseases.Infectious.Diseases.Epithelial.cell.signaling.in.Helicobacter.pylori.infection	0.046**
Metabolism.Metabolism.of.Cofactors.and.Vitamins.Nicotinate.and.nicotinamide.metabolism	0.074*
Metabolism.Metabolism.of.Cofactors.and.Vitamins.One.carbon.pool.by.folate	0.093*
Metabolism.Nucleotide.Metabolism.Pyrimidine.metabolism	0.093*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Drug.metabolism...other.enzymes	0.093*
Unclassified.Metabolism.Glycan.biosynthesis.and.metabolism	0.074*
Unclassified.Cellular.Processes.and.Signaling.Cell.division	0.036**
Metabolism.Metabolism.of.Cofactors.and.Vitamins.Vitamin.B6.metabolism	0.059*
Metabolism.Metabolism.of.Other.Amino.Acids.D.Glutamine.and.D.glutamate.metabolism	0.093*
Metabolism.Energy.Metabolism.Methane.metabolism	0.059*
Metabolism.Biosynthesis.of.Other.Secondary.Metabolites.Novobiocin.biosynthesis	0.074*
Metabolism.Amino.Acid.Metabolism.Phenylalanine..tyrosine.and.tryptophan.biosynthesis	0.074*
Human.Diseases.Metabolic.Diseases.Type.I.diabetes.mellitus	0.093*
Human.Diseases.Metabolic.Diseases.Type.II.diabetes.mellitus	0.093*

**Table S1. (Continued)**

<b><i>Week 4 data- higher in RW- Cohort</i></b>	<b><i>P-Value</i></b>
Human.Diseases.Neurodegenerative.Diseases.Amyotrophic.lateral.sclerosis..ALS.	0.046**
Metabolism.Amino.Acid.Metabolism.Lysine.biosynthesis	0.046**
Genetic.Information.Processing.Translation.Translation.factors	0.074*
Genetic.Information.Processing.Transcription.Basal.transcription.factors	0.009**
Genetic.Information.Processing.Replication.and.Repair.DNA.replication.proteins	0.093*
Genetic.Information.Processing.Replication.and.Repair.Homologous.recombination	0.093*
Genetic.Information.Processing.Folding..Sorting.and.Degradation.Protein.export	0.093*
Cellular.Processes.Transport.and.Catabolism.Peroxisome	0.036**
Cellular.Processes.Cell.Growth.and.Death.Cell.cycle...Caulobacter	0.093*
<b><i>Week 8 data- Higher in RW+ Cohort</i></b>	
Environmental.Information.Processing.Signaling.Molecules.and.Interaction.Ion.channels	0.074*
Human.Diseases.Infectious.Diseases.African.trypanosomiasis	0.074*
Metabolism.Amino.Acid.Metabolism.Tyrosine.metabolism	0.059*
Metabolism.Lipid.Metabolism.Linoleic.acid.metabolism	0.021**
Metabolism.Metabolism.of.Cofactors.and.Vitamins.Retinol.metabolism	0.093*
Metabolism.Metabolism.of.Other.Amino.Acids.Glutathione.metabolism	0.074*
Metabolism.Xenobiotics.Biodegradation.and.Metabolism.Chlorocyclohexane.and.chlorobenzene.degradation	0.093*
Organismal.Systems.Immune.System.RIG.I.like.receptor.signaling.pathway	0.093*
<b><i>Week 8 data- Higher in RW- Cohort</i></b>	
Genetic.Information.Processing.Transcription.Basal.transcription.factors	0.021**
Human.Diseases.Infectious.Diseases.Vibrio.cholerae.pathogenic.cycle	0.074*
Human.Diseases.Neurodegenerative.Diseases.Parkinson.s.disease	0.074*
Metabolism.Energy.Metabolism.Methane.metabolism	0.046**
Unclassified.Metabolism.Glycan.biosynthesis.and.metabolism	0.027**



**Figure S1. Plasma hormone levels**



## **Chapter 5**

**Short term exercise and/or dietary protein supplementation do not introduce elite athlete-like gut microbial diversity in previously inactive individuals.**

### **Peter Skuse Chapter Contributions**

#### **Experimental:**

- Designed and performed all experiments relating all fecal sample DNA extraction, PCR amplification and DNA sequencing

#### **Results interpretation:**

- Analysis of all data pertaining to 16S rRNA gene sequencing.

## 5.1 ABSTRACT

The gut microbiota of professional rugby players was recently shown to be significantly more diverse than that of controls. This increased diversity correlated with creatine kinase levels, a marker of extreme exercise, and dietary protein intake. To investigate the possibility of a link between exercise, protein consumption and the gut microbiota, the current study employed a three arm strategy to evaluate the impact of 1) an eight week exercise program (Ex), 2) the same exercise program in combination with daily whey protein supplementation (Ex+WPS) and 3) eight weeks of daily whey protein supplementation without exercise (WPS) on the composition and diversity of the gut microbiota of physically inactive subjects. Over the eight week intervention period, exercise increased the general fitness of the volunteers but did not significantly alter the composition or diversity of the gut microbiota. Within the Ex+WPS and WPS cohorts, a significant increase in *Lactococcus* populations was apparent, which is assumed to be as a consequence of daily whey protein supplementation. These results establish that eight week exercise regimes or dietary whey protein supplementation have negligible to subtle impacts on the gut microbiota and, thus, longer term intervention studies are required to determine if more considerable changes can be induced by these interventions.

## 5.2 INTRODUCTION

The human gut microbiota has been the subject of intensive investigation over the last decade, largely due to increasing number of studies linking its composition and function with health and disease. A ‘normal/healthy-functioning’ gut microbiota has been shown to confer several health benefits to the host, such as protection against opportunistic pathogens and modulation of the host immune system (Sekirov et al. 2010, Joyce and Gahan 2014). Conversely, perturbations in the composition of the normal gut microbiota have been implicated in the pathogenesis of several disease states, including type-2-diabetes, obesity and inflammatory bowel disease (Qin et al. 2012, Moran and Shanahan 2014, Marchesi et al. 2015). Therefore the gut microbiota may be thought of as an environmental factor which may confer risk or resistance to a wide range of inflammatory and metabolic disorders.

Changes in human behaviour have, in turn, been shown to invoke changes to the composition of the gut microbiota (Shanahan 2012). Several studies have shown that changes in dietary habits can result in alterations in the composition of the gut microbiota. Walker *et al.* reported a change in the fecal bacterial populations in obese men within four days after the subjects had been switched to a diet supplemented with resistant starch (Walker et al. 2011). Furthermore, Claesson *et al.* reported that community dwelling elderly subjects which consumed a healthier and more diverse diet, harboured a more diverse gut microbiota than individuals residing in long term care facilities which consumed a less diverse diet (Claesson et al. 2011). Although exercise is also an environmental factor that influences a wide range of physiological processes, its impact on the gut microbiota has received relatively little attention. This is despite data showing that the type and degree of exercise undertaken by an individual invokes differential impacts on intestinal function.

Moderate exercise is associated with an improvement in intestinal barrier function and reduced bacterial translocation (Luo et al. 2014). In mice, forced aggressive treadmill training has been shown to aggravate inflammatory indices and outcomes in colitis-induced mice, while conversely, voluntary running wheel exercise produced a protective effect (Cook et al. 2013). Given the capacity for exercise to influence intestinal conditions, it is a reasonable supposition that physical activity also influences commensal enteric microbial populations. It is also notable that exercise has been increasingly recognised as an excellent alternative to pharmaceutical treatments for several disease states that have been linked with the gut microbiota, such as type-2-diabetes (Schwingshackl et al. 2014), irritable bowel syndrome (IBS) (Johannesson et al. 2011) and colorectal cancer (CRC) (Robsahm et al. 2013). To date, the majority of the relatively few studies that have investigated the influence of physical activity on the enteric microbiota have utilised rodent models, and have tended to explore the impact of physical activity on the intestinal microbiota in combination with dietary interventions such as low or high fat diets (Evans et al. 2014, Allen et al. 2015). In one recent human study, Clarke *et al.* reported that professional rugby players harboured a gut microbiota which was significantly more diverse, and had a significantly lower inflammatory tone, than that of high or low BMI control subjects. Moreover, this increased biodiversity significantly and positively correlated with increased dietary protein intake and creatine kinase (serving as a proxy for the degree of exercise), raising the question; are exercise and increased protein intake drivers of microbial diversity within the gut (Clarke et al. 2014)?

To address this topic, this study aimed, through the use of high-throughput DNA sequencing, to determine if an eight week exercise intervention and/or whey protein

supplementation would influence the gut microbiota of previously physically inactive individuals whilst maintaining habitual dietary habits.

## **5.3 MATERIALS AND METHODS**

### **5.3.1 Ethical Approval and informed consent**

Prior to commencement of the study, ethical approval was sought and granted by the Clinical Research Ethics Committee (CREC) of the Cork Teaching Hospitals, Cork City, Ireland, in accordance with guidelines outlined in the declaration of Helsinki. All volunteers provided informed consent prior to their participation in the study and were informed of their right to withdraw consent at any stage.

### **5.3.2 Subject recruitment**

Healthy males and females between the ages of 18 and 40 years with a body mass index (BMI) of 22-35 were recruited subject to meeting the inclusion/exclusion criteria outlined in table 1. At the time of recruitment, individuals were required to be physically inactive as determined using the short form of the International Physical Activity Questionnaire. Volunteers with significant medical histories that posed a potential harm to the patient by participating in an exercise program were excluded. All participants were screened for potential risks using an adapted version of the American College of Sports Medicine's safe participation guidelines (Pescatello 2014).

### **5.3.3 Withdrawal and exclusion from the study**

Less than 90% compliance with WPS led to exclusion from the study. Likewise, participants who missed over one week of the study period due to any reason were excluded from the study. Adverse events experienced by the subjects during the study were recorded and discussed with the principal investigators as to whether it

was safe for the participant to continue. Any subjects experiencing a serious adverse event during the study period was to be withdrawn.

#### **5.3.4 Experimental Design**

This study comprised a prospective, randomized, three armed, diet-controlled trial based in a single centre at Cork University Hospital, Cork, Ireland. Volunteers assigned to arm 1 of the study (Ex) participated in a defined exercise program throughout the 8 week intervention period (n=26). Volunteers assigned to arm 2 of the study (Ex+WPS) undertook the same 8-week exercise program in combination with a once daily whey protein supplement (WPS) for the duration of the study period (n=22). Finally participants assigned to arm 3 of the study (WPS) only consumed the daily WPS and did not participate in the exercise program (n=27).

Baseline (week 0) and post intervention (week 8) anthropometric characteristics were recorded for each subject recruited into the study (Table 2). Resting blood pressure and body composition were also recorded at the same time points. Blood pressure was measured in the seated position using a calibrated electronic sphygmomanometer (Welch-Allyn, 300 Series, Beaverton, OR, USA). Dual Energy X-ray absorptiometry (DEXA) was used to assess body composition using a GE Healthcare Lunar iDXA (GE-Healthcare.). Total body scans of the participants were performed using the same apparatus providing whole body and segmental analyses (trunk, arms and legs). The enCORE software (version 13.4, 2010) was used for scan analysis and uses a 3-compartment model to estimate body fat percentage %BF. Total body fat values were derived using the region %BF value calculated from the GE Lunar iDXA machine ( $\text{Region \%BF} = \frac{\text{Fat mass}}{\text{Fat} + \text{Lean} + \text{Bone mass}}$ ). Apparatus calibration was performed before the commencement of the study period



and quality control assessment was performed before each scanning session.

### **5.3.5 Blood and stool sampling**

Approximately 10 – 15mls of venous blood was drawn per participant at the same previously mentioned time points. Blood was filled into standard EDTA and serum blood tubes extensive blood profile testing was carried out at the Mercy University Hospital Haematology and Biochemistry laboratories (Table 3). Commercial multiplex microplates (Meso Scale Diagnostics, Rockville, Maryland, USA) were used to measure volunteers resting cytokine profiles. All participants provided a fresh stool sample at the week 0 and week 8 time points for total genomic DNA extraction and subsequent Illumina Miseq compositional sequencing.

### **5.3.6 Whey protein supplementation**

Subjects randomized to the Ex+WPS or WPS groups were required to take a once daily whey protein supplement (WPS) (30g) (Carbery foods, Ballineen, Co. Cork). Throughout the study period, participants were asked to maintain their normal dietary patterns and asked to refrain from taking unspecified dietary, multivitamin or herbal. Subjects already consuming dietary supplements prior to recruitment underwent a 14 day washout period before participating in the study.

### **5.3.7 Structured exercise program**

All subjects undertook an 8-week mixed aerobic and resistance training program having first undergone a 90 minute induction session with a qualified gym instructor. Subjects were required to train 3 times per week aiming for a total of 24 sessions during the 8-week study period. All sessions took place in the Mardyke Arena gymnasium at University College Cork, Cork, Ireland.

Aerobic exercise was standardized and progressive, suitable for inactive and exercise naïve individuals. The program required similar levels of energy expenditure to couch to 5km training regimes. Subjects were allowed to select from a number of cardiovascular machines (treadmill, upright stationary bike, cross-trainer and stepper machine). Duration of activity was altered to ensure that similar levels of energy expenditure occurred regardless of choice of machine. Aerobic activity was of moderate to vigorous intensity (5 to 7/10 on the Borg scale of perceived exertion).

At resistance each training session, participants were required to complete 7 out of 10 resistance exercises including 3 upper body, 3 lower body and 1 core muscle exercise. Starting weights were 70% of the individual's one repetition maximum valued calculated using the Brzycki formula (Brzycki 1998). Free weight use was not permitted in the study. Progress, attendance and compliance to the subject's training programs recorded using the FitLinxx system installed in all machines in the Mardyke arena.

### **5.3.8 Cardio-respiratory fitness testing**

Baseline and post intervention cardio-respiratory fitness levels among subjects were measured using the 1-mile walk test (Rockport test) as described by Kline (Kline et al. 1987). This validated measure is a sub-maximal aerobic fitness test that predicts VO<sub>2</sub> maximum values based on subject's weight, age, sex, time to complete a brisk one mile walk and heart rate upon completion of the walk. A sub-maximal fitness test was chosen for participant safety. Each participant was allowed a 3 to 5 minute warm up before commencement of the test. The test was performed at a temperature controlled indoor track at the Mardyke Arena.

### **5.3.9 DNA extraction**

Total genomic DNA was extracted from fresh (within 2 hours after excretion) stool samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) which was combined with an additional bead beading step (30s x 3). Extracted DNA was quantified using the Nanodrop 1000 spectrophotometer and stored at -20°C.

### **5.3.10 Illumina MiSeq 16S rRNA amplicon sequencing**

The V3-V4 variable region of the 16S rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina, San Diego CA, USA). Initially the template DNA was amplified using primers specific to the V3-V4 region of the 16S rRNA gene which also allowed for the Illumina overhang adaptor, where, the forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse primer (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used. Each PCR reaction contained 2.5µl DNA template, 5 µl forward primer (1µM), 5 µl reverse primer (1µM) (Sigma, Ireland) and 12.5 µl Kapa HiFi Hotstart Readmix (2X) (KAPA Biosystems Inc, USA). The template DNA was amplified under the following PCR conditions for a total of 25 cycles: 95°C for 3 minutes and 30 seconds respectively (initialization and denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (elongation) followed by a final elongation period of 5 minutes. A negative control reaction whereby the template DNA was replaced with PCR grade water was employed to confirm lack of contamination and PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose gel, 100v) post PCR reaction.

Successful amplicons were then cleaned using the AMPure XP purification system (Labplan, Dublin, Ireland). A second PCR reaction was then completed using the previously amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, CA, USA) were used per sample to allow all samples to be pooled, sequenced on one flow cell and subsequently identified bioinformatically. Each reaction contained 25 µl Kapa HiFi HotStart ReadyMix (2X), 5 µl template DNA, 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx) and 10 µl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just 8 cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 fluorometer (Invitrogen) in conjunction with the broad range DNA quantification assay kit (Life technologies, Carlsbad, CA, USA). All samples were then pooled to an equimolar concentration and the pool underwent a final cleaning step. Quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2M NaOH, diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX. Samples were then sequenced in house on the MiSeq sequencing platform using a 2.300 cycle V3 Kit following protocols outlined by Illumina.

#### **5.3.11 Bioinformatic analysis**

Three hundred base pair paired-end reads were assembled using FLASH (fast length adjustment of short reads to improve genome assemblies). Paired-end reads were further processed with the inclusion of quality filtering based on a quality score of > 25 followed by subsequent removal of mismatched barcodes and sequences below length threshold using QIIME. USEARCH v7 (64-bit) was then used for denoising

and chimera detection as well as clustering into operational taxonomic units (OTUs). PyNAST was used to align OTUs and taxonomy was assigned by using BLAST against the SILVA SSURef database release 111. QIIME was used to generate Alpha and Beta diversities, calculated based on Bray Curtis, weighted and unweighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were then visualised using EMPERor v0.9.3-dev. Predictive functional profiling was performed using the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) tool (Langille et al. 2013).

### **5.3.12 Statistical analysis**

All statistical analysis was carried out using the R statistical package (V.2.13.1). A paired t-test was employed to compare baseline (week 0) and post intervention (week 8) blood cytokine levels and anthropometric measurements, where statistical significance was taken as  $p \leq 0.05$ . The non-parametric Wilcoxon test was used to determine significant differences in microbial taxonomies,  $\alpha$ -diversity indices and PICRUSt predicted metabolic pathways between baseline and post intervention sequence data where statistical significance was again taken as  $p \leq 0.05$ . Spearman correlations were used to examine relationships between the measured parameters where significance was accepted as  $p < 0.05$

## **5.4 RESULTS**

### **5.4.1 An eight week structured exercise programme improves subject's fitness and blood pressure profile**

Subject's anthropometric characteristics are summarised in table 2. When subject's post intervention (week 8) anthropometric measurements were compared to their baseline (week 0) measurements,  $\text{VO}_2$  max was determined to be significantly higher in individuals assigned to the Ex ( $p=0.001$ ) and Ex+WPS ( $p=0.006$ ) groups. Diastolic pressure was significantly reduced in the Ex ( $p=0.016$ ), Ex+WPS ( $p=0.040$ ) and WPS ( $p=0.024$ ) groups, with a significant reduction in systolic pressure also noted in the Ex+WPS ( $p=0.005$ ) and WPS ( $p=0.039$ ) groups (Figure 2). No other significant differences in anthropometric characteristics were observed.

### **5.4.2 A combination of exercise and whey protein supplementation improves inflammatory tone**

There were no significant differences between the baseline and post intervention blood profiles of either the Ex or WPS cohorts. However, when compared to baseline measurements, subjects within the Ex+WPS cohort displayed significantly lower levels of IL-8 ( $p=0.039$ ) after the intervention period (Figure 3).

### **5.4.3 Exercise, whey protein supplementation or a combination of both does not alter the diversity of the gut microbiota**

Faecal samples were collected before and after the intervention period. After DNA extraction and amplicon generation, a total of 15,161,178 16S rRNA post-quality paired end sequence reads were generated during the sequencing process, corresponding to an average of 101,075 reads per individual time point. Baseline and

post intervention alpha diversity values were calculated for species richness (Chao1), biodiversity (Shannon index), observed species and species number relative to abundance within the sample (Simpson diversity index) in each case. Comparison of the baseline and post intervention values for each of the calculated indices revealed no significant differences in alpha diversity among any of the three test cohorts (Figure 4).

Spearman correlations were used to examine the relationships between week 8 assigned alpha diversity values and post intervention clinical data (Figure 5). In the Ex cohort, alkaline phosphatase was noted to negatively correlate with Shannon index ( $r^2 = -0.638$ ,  $p < 0.001$ ), Chao1 ( $r^2 = -0.530$ ,  $p = 0.005$ ), Simpson index ( $r^2 = -0.573$ ,  $p = 0.002$ ) and observed species ( $r^2 = -0.557$ ,  $p = 0.003$ ), while lean mass was positively correlated with Chao1 ( $r^2 = 0.418$ ,  $p = 0.034$ ). In the Ex+WPS cohort, Shannon index was negatively correlated with % change in body fat ( $r^2 = -0.460$ ,  $p = 0.031$ ) and positively correlated with % increase in lean mass ( $r^2 = 0.429$ ,  $p = 0.046$ ). Finally, in the WPS cohort, negative correlations were observed between alkaline phosphatase and Chao1 ( $r^2 = -0.471$ ,  $p = 0.013$ ), PD whole tree ( $r^2 = -0.495$ ,  $p = 0.009$ ) and observed species ( $r^2 = 0.473$ ,  $p = 0.021$ ).

Principal coordinate analysis (PCoA), based upon unweighted Unifrac distances, revealed proximal clustering, i.e. no clear separation, between the baseline and post intervention time points for each of the three test groups within the study (Figure 6). Similarly, Procrustes imaging of unweighted Unifrac distances matrices revealed no uniform change in beta diversity between baseline and post intervention samples (Figure 7).

#### **5.4.4 A short period of exercise, whey protein supplementation or a combination of both does not invoke major changes to gut microbiota taxonomy**

Phylogenetic analysis was examined at phylum, family and genus level for each of the study's three cohorts to determine if changes in microbial taxonomy occurred during the intervention period (Figure 8). No significant phylogenetic differences were determined within the gut microbiotas of individuals assigned to the Ex group from week 0 to week 8. *Lactococcus* proportions were determined to be significantly increased from week 0 to week 8 in the Ex+WPS ( $p=0.014$ ) and WPS ( $p=0.002$ ) cohorts, while proportions of the *Veillonellaceae* ( $p=0.024$ ) family and *Marvinbryantia* ( $p=0.035$ ) genus significantly increased and decreased, respectively, from week 0 to week 8 in the WPS cohort. Similarly PICRUSt analysis of predicted functional pathways revealed no significant differences between baseline and post intervention data for any of the three groups of the study.

#### **5.4.5 High compliance with exercise program may differentially alter microbial taxonomies within the gut microbiota**

To determine if the amount and type of exercise completed by the subjects within the Ex and Ex+WPS groups correlated with changes in the diversity or composition of the gut microbiota, phylogenetic comparative analysis of baseline and post intervention sequence data of eight individuals which completed the most or least amount of either cardiovascular (as determined by total minutes of cardiovascular training) or resistance training (as determined by total repetitions completed) in each group (Figure 9). Throughout the study period, individuals within the Ex group which completed the most cardiovascular training were noted to have increased



proportions of *Gordonibacter* ( $p=0.032$ ), while those completing the most resistance training had increased proportions of *Succinivibrionaceae/Succinivibrio* ( $p=0.035$ ) from week 0 to week 8. No significant differences were observed between week 0 and week 8 data for individuals within the Ex+WPS group which completed the most cardiovascular training. However those which completed the most resistance training showed significantly lower proportions of *Actinomycetaceae/Actinomyces* ( $p=0.05$ ) and significantly increased *Eggerthella* proportions ( $p=0.032$ ).

## 5.5 DISCUSSION

The gut microbiota of professional athletes has previously been shown to be significantly more diverse than that of control subjects. This increased diversity was positively correlated with exercise and protein intake levels (Clarke et al. 2014), suggesting that further studies to investigate the possibility of a link between diet and/or exercise with gut microbial diversity were merited. Therefore this study, through a three armed approach, aimed to evaluate the impact of exercise, increased dietary protein and the combined effect of both on the composition and diversity of the gut microbiota in physically inactive individuals over a short-term intervention period.

An improvement in aerobic fitness was observed in individuals assigned to the Ex and Ex+WPS groups, as determined by a significant increase from baseline to post intervention in  $\text{VO}_2$  measurements. Regular exercise is well known to reduce blood pressure and, consistent with this, subjects assigned to both Ex and Ex+WPS groups displayed a significantly lower resting blood pressure after the intervention period. Notably, a significant reduction between baseline and post intervention resting blood pressure was also observed in the WPS cohort. This reduction is likely due to the anti-hypertensive properties which several whey derived peptides have been shown to exert *via* angiotensin-converting enzyme (ACE) inhibition (Pihlanto-Leppälä 2000, Pal and Ellis 2010).

In their study of professional athletes, Clarke *et al.* showed that, when compared to control cohorts, the athlete cohort had a significantly higher dietary protein intake and a significantly lower inflammatory tone (Clarke et al. 2014). In the present study, circulating levels of the pro-inflammatory cytokine IL-8 were significantly

reduced throughout the intervention period in those individuals assigned to the Ex+WPS group. This reduction in IL-8 levels suggests that even a short term combination of exercise and whey protein supplementation lowers individuals inflammatory tone which in turn could have beneficial implications for inflammatory disease states such as type-2-diabetes, obesity and IBD (Murata et al. 1995, Kim et al. 2006).

With respect to the subjects' gut microbiota, no significant change in alpha diversity was observed throughout the intervention period for any of the three groups (Figure 4). The intervention period employed may have been too short to induce a substantial change to the biodiversity of the gut microbiota. Population studies have shown that enteric microbial diversity may be greatly influenced by long term dietary habits, for example children from a rural African village, which consume a diet high in polysaccharides, were observed to harbour a significantly more diverse microbiota than that of European children consuming a typical 'western' diet (De Filippo et al. 2010). While, with respect to whey protein supplementation, we have previously shown that even over a long intervention period (21 weeks), whey protein supplementation did not increase the microbial biodiversity of the murine gut microbiota (McAllan et al. 2014). Consistently, murine studies which investigated the effect of exercise on the gut microbiota over a comparable intervention period have also reported that exercise does not invoke increases in alpha diversity measures (Chapter 4) (Allen et al. 2015). Furthermore, human exercise intervention studies have shown that a longer period of regular exercise (> 6 months) is required to invoke changes to host physiology which in turn may result in alterations to the diversity of the gut microbiota (Balducci et al. 2010, Ho et al. 2013). Similarly, beta diversity based upon unweighted Unifrac distances (Figure 5) showed no clear

separation between baseline and post intervention data for each of the three groups within the study. The lack of separation observed between pre and post intervention data points for individuals consuming a daily WPI supplement might be unexpected as we have previously shown, in murine models, that whey protein consumption resulted in distinct beta diversity clustering patterns, even over a short intervention period ((McAllan et al. 2014); Chapter 3). The lack of separation seen presently likely arises from the short intervention period combined with the large inter-individual dietary variations, the latter being an issue not encountered when dealing with laboratory animals (Nguyen et al. 2015). The proximal clustering observed within the Ex cohort is consistent with rodent studies examining the effect of voluntary exercise on the gut microbiota over a comparable period, further suggesting that exercise does not invoke substantial changes to the gut microbiota over a short intervention period (Chapter 4)(Allen et al. 2015).

With respect to phylogenetic analysis, no significant differences were observed throughout the intervention period in individuals assigned to the Ex groups, once again highlighting that while exercise may increase physical fitness over a short period, a longer intervention period may be required to invoke changes to gut microbial populations. When compared to the trial's start point, *Lactococcus* proportions were significantly increased post intervention in both Ex+WPS and WPS cohorts. Members of the *Lactococcus* genera are naturally found in dairy products, in addition to their use as dairy starter cultures, and have been shown to possess potent proteolytic activity (Korhonen and Pihlanto 2006). Thus the significant increase in *Lactococcus* observed in the Ex+WPS and WPS groups presumably arises directly from the consumption of lactococci within the WPS with lactococci acquired from other dietary sources also potentially benefiting from the

ability of members of this bacterial genus to utilise the proteins within whey as a growth source. While it would appear that whey protein intake increases the proportions of lactococci, further investigation is required examining the viability of lactococci cells present in whey and in turn wither this increase in lactococci impacts the host. *Veillonellaceae* were also significantly increased in the WPS cohort throughout the intervention period. Members of the *Veillonellaceae* family have been shown to preferentially ferment amino acids (Scott et al. 2013). Furthermore, we have previously shown (Chapter 3) that the whey protein lactoferrin enriched populations of *Veillonellaceae* whose increased proportions may contribute to health *via* their conversion of lactate to the short chain fatty acid propionate (Flint et al. 2012, Smith et al. 2013).

Spearman correlations between anthropometric measurements and alpha diversity indices revealed a positive correlation between lean mass and % change in lean mass in the Ex (Chao1) and Ex+WPS (Shannon) groups, respectively. Given that the gut microbiota of obese individuals has been reported to exhibit reduced biodiversity (Turnbaugh et al. 2008), these present results suggest that exercise may drive changes in body composition which in turn increases the diversity of the gut microbiota..

Finally, to assess if the amount and type of exercise completed by subjects correlated with specific changes in enteric microbial populations, baseline and post intervention sequence data was compared for individuals which completed the most and least amount of exercise throughout the eight week period. Within the Ex cohort, subjects which completed the most resistance training displayed a significant increase in the proportions of *Succinivibrionaceae/Succinivibrio* were noted. Higher proportions of *Succinivibrionaceae/Succinivibrio* were also evident when the elite

rugby athletes microbiota was compared to either high or low BMI controls (Clarke et al. 2014). Similarly, subjects assigned to the EX+WPS group, which completed the most resistance training, showed a significant reduction from week 0 to week 8 in the proportions of *Actinomycetaceae/Actinomyces*, which have previously been associated with inflammatory disease states such as obesity and IBD (Frank et al. 2007, Mandal et al. 2015). Results from these subsets within the groups of our study suggest that more intensive exercise, particularly resistance training, may influence the gut microbiota.

Results from the present study therefore are consistent with both our own previous work and that of other studies and further suggests that, while exercise and dietary protein supplementation can improve individual's fitness over a short period, a longer intervention period is required to determine if considerable alterations to the gut microbiota can be brought about.

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**Table 1. Subject recruitment inclusion/exclusion criteria**

Inclusion/exclusion criteria
Personal history of coronary artery disease, congenital heart disease or any cardio-vascular disease
Family history of known coronary artery disease before 45 years of age
Uncontrolled hypertension (>140/90mmHg)
Known renal or hepatic impairment
Type 1 or type 2 diabetes mellitus
Significant pulmonary disease – not including well-controlled, mild asthma
Primary or secondary immunodeficiency or autoimmune disorder
Current smoker or ex-smoker of less than 3 months duration.
Psychiatric disorders including previous history of depression
A history of substance abuse
Current or recent involvement in another clinical research study
Known or suspected hypersensitivity to the dietary supplementation
Gastro-intestinal disease e.g. coeliac disease, inflammatory bowel disease, irritable bowel syndrome or significant gastro-intestinal surgery
Previous significant gastro-intestinal surgery e.g. colectomy
Any other medical condition deemed exclusionary by the investigators including suspected or confirmed pregnancy

**Table 2. Anthropometric characteristics**

	Ex (W0)	Ex (W8)	Ex+WPS (W0)	Ex+WPS (W8)	WPS (W0)	WPS (W8)
<b>BMI</b>	27.98 ± 0.68	27.58 ± 0.64	27.90 ± 0.74	27.61 ± 0.77	27.35 ± 0.63	27.27 ± 0.68
<b>Waist/Hip Ratio</b>	0.86 ± 0.01	0.85 ± 0.01	0.85 ± 0.02	0.84 ± 0.02	0.83 ± 0.01	0.84 ± 0.01
<b>Systolic Pressure</b>	126.46 ± 2.43	121.54 ± 1.88	<b><i>126.86 ± 2.35</i></b>	<b><i>118.18 ± 1.73</i></b>	<b><i>124.33 ± 1.68</i></b>	<b><i>118.67 ± 2.08</i></b>
<b>Diastolic Pressure</b>	<b><i>80.27 ± 1.69</i></b>	<b><i>74.65 ± 1.50</i></b>	<b><i>79.95 ± 2.12</i></b>	<b><i>71.73 ± 1.25</i></b>	<b><i>79.00 ± 1.15</i></b>	<b><i>74.67 ± 1.46</i></b>
<b>Heart Rate</b>	72.88 ± 1.95	68.19 ± 1.85	69.00 ± 2.34	65.14 ± 1.91	72.59 ± 1.81	75.96 ± 1.79
<b>Total % Body Fat</b>	33.32 ± 1.25	31.99 ± 1.23	33.93 ± 1.35	32.96 ± 1.37	34.78 ± 1.44	35.20 ± 1.47
<b>Fat Mass (Kg)</b>	28.47 ± 1.74	27.40 ± 1.79	28.46 ± 1.86	27.65 ± 1.86	27.31 ± 1.51	27.63 ± 1.57
<b>Total % Lean Mass</b>	63.15 ± 1.20	64.46 ± 1.18	62.62 ± 1.29	63.50 ± 1.32	61.73 ± 1.38	61.39 ± 1.41
<b>Lean Muscle Mass (Kg)</b>	52.42 ± 2.22	53.20 ± 2.22	1.97 ± 2.69	52.37 ± 2.61	47.91 ± 1.52	47.64 ± 1.50
<b>VO<sub>2</sub> Max</b>	<b><i>41.82 ± 1.12</i></b>	<b><i>46.88 ± 0.085</i></b>	<b><i>42.42 ± 0.98</i></b>	<b><i>47.11 ± 1.31</i></b>	N/A	N/A
<b>Rockport HR</b>	129.27 ± 4.58	128.00 ± 3.76	132.32 ± 4.06	128.27 ± 3.64	N/A	N/A

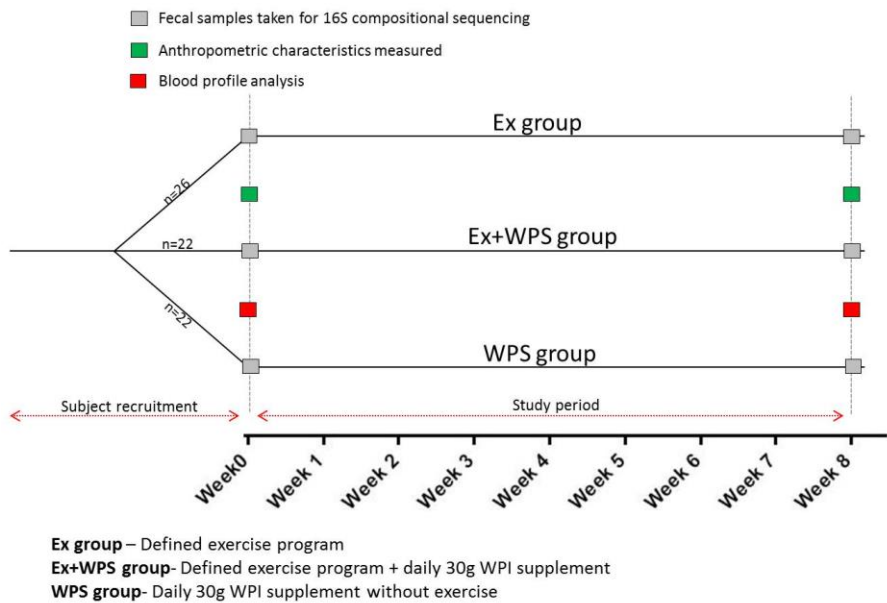
Data shown as mean ± SEM where statistically significant differences between baseline and post intervention data are highlighted in bold and italics ( $p \leq 0.05$ )

**Table 3. Blood profile analysis**

	Ex (W0)	Ex (W8)	Ex+WPS (W0)	Ex+WPS (W8)	WPS (W0)	WPS (W8)
Hgb	14.57 ± 0.25	14.36 ± 0.26	14.69 ± 0.32	14.24 ± 0.34	13.93 ± 0.26	13.66 ± 0.27
RBC	5.04 ± 0.1	4.94 ± 0.11	5.02 ± 0.11	4.83 ± 0.11	4.83 ± 0.09	4.69 ± 0.09
Hct	0.44 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
Platelets	270.2 ± 10.71	260.66 ± 10.47	264.78 ± 14.57	246.1 ± 12.45	276.49 ± 11.67	247.78 ± 10.67
WBC	6.48 ± 0.31	6.47 ± 0.4	6.33 ± 0.26	5.84 ± 0.3	6.21 ± 0.23	6.58 ± 0.31
Neuts	3.44 ± 0.21	3.51 ± 0.27	3.55 ± 0.18	3.05 ± 0.22	3.55 ± 0.18	3.95 ± 0.29
Lymph	2.25 ± 0.14	2.19 ± 0.16	2.07 ± 0.09	2.1 ± 0.12	1.87 ± 0.09	1.86 ± 0.08
Monocytes	0.44 ± 0.04	0.43 ± 0.04	0.39 ± 0.02	0.4 ± 0.03	0.42 ± 0.03	0.42 ± 0.03
Eosinophils	0.22 ± 0.04	0.23 ± 0.04	0.18 ± 0.03	0.18 ± 0.03	0.24 ± 0.03	0.22 ± 0.03
Basophils	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Na	140.16 ± 0.43	139.54 ± 0.31	140.19 ± 0.44	139.46 ± 0.36	139.88 ± 0.38	139.82 ± 0.32
K	4.55 ± 0.06	4.49 ± 0.07	4.57 ± 0.09	4.54 ± 0.07	4.7 ± 0.13	4.56 ± 0.07
Urea	5.39 ± 0.25	5.32 ± 0.29	5.05 ± 0.28	5.03 ± 0.31	4.89 ± 0.17	5.42 ± 0.25
Creatinine	80.43 ± 2.94	81.77 ± 2.73	76.1 ± 2.68	76.96 ± 2.97	73.56 ± 1.7	72.00 ± 1.65
AST	19.97 ± 1.1	20.39 ± 1.24	21.87 ± 1.73	18.6 ± 0.76	20.8 ± 2.07	21.75 ± 1.83
GGT	26.47 ± 3.27	26.43 ± 5.43	28.78 ± 4.18	23.32 ± 3.13	24.3 ± 2.42	22.34 ± 1.89
CK	110.97 ± 11.45	124.81 ± 19.02	172.41 ± 64.23	126.19 ± 20.31	103.23 ± 8.5	110.75 ± 10.59
Alk Phos	63.31 ± 3.86	64.00 ± 3.89	56.19 ± 2.83	56.96 ± 2.52	61.67 ± 2.87	60.38 ± 2.5
Calcium	2.39 ± 0.02	2.37 ± 0.02	2.38 ± 0.02	2.37 ± 0.02	2.42 ± 0.02	2.39 ± 0.02
Phosphate	1.11 ± 0.04	1.08 ± 0.04	1.08 ± 0.04	1.07 ± 0.04	1.25 ± 0.05	1.15 ± 0.04
CRP	2.93 ± 0.74	2.54 ± 0.57	2.28 ± 0.57	3.6 ± 1.38	1.67 ± 0.26	2.19 ± 0.63
IFN	13.47 ± 2.65	12.34 ± 3.17	7.4 ± 0.63	11.45 ± 3.02	6.74 ± 0.74	8.11 ± 1.63
IL-10	0.39 ± 0.05	0.74 ± 0.45	0.46 ± 0.07	1.22 ± 0.79	0.34 ± 0.09	0.41 ± 0.1
IL-12 p70	0.9 ± 0.45	1.83 ± 1.6	0.74 ± 0.26	3.00 ± 2.07	0.5 ± 0.16	0.7 ± 0.31
IL-1beta	0.25 ± 0.11	0.2 ± 0.14	0.14 ± 0.06	0.56 ± 0.46	0.04 ± 0.02	0.13 ± 0.07
IL-6	0.83 ± 0.24	0.95 ± 0.42	0.75 ± 0.17	1.38 ± 0.77	0.56 ± 0.09	0.78 ± 0.18
IL-8	33.48 ± 8.05	80.4 ± 49.49	<b>82.16 ± 23.5</b>	<b>28.21 ± 9.27</b>	10.73 ± 0.84	82.27 ± 41.82
TNF	2.96 ± 0.27	3.1 ± 0.61	3.61 ± 0.63	3.41 ± 0.58	2.16 ± 0.1	2.43 ± 0.17

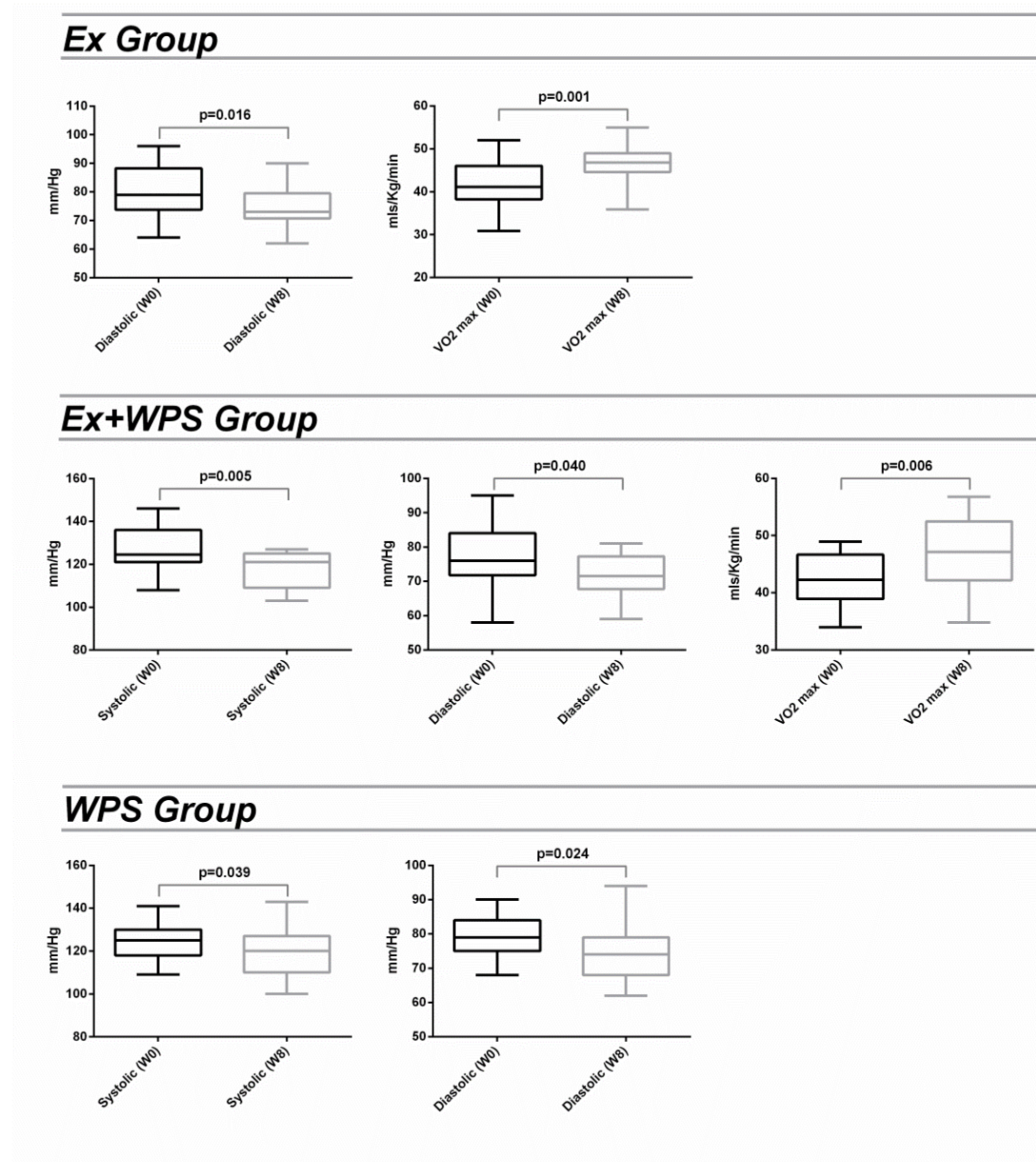
Data shown as mean ± SEM where statistically significant differences between baseline and post intervention data are highlighted in bold and italics ( $p \leq 0.05$ )

**Figure 1. Experimental overview**



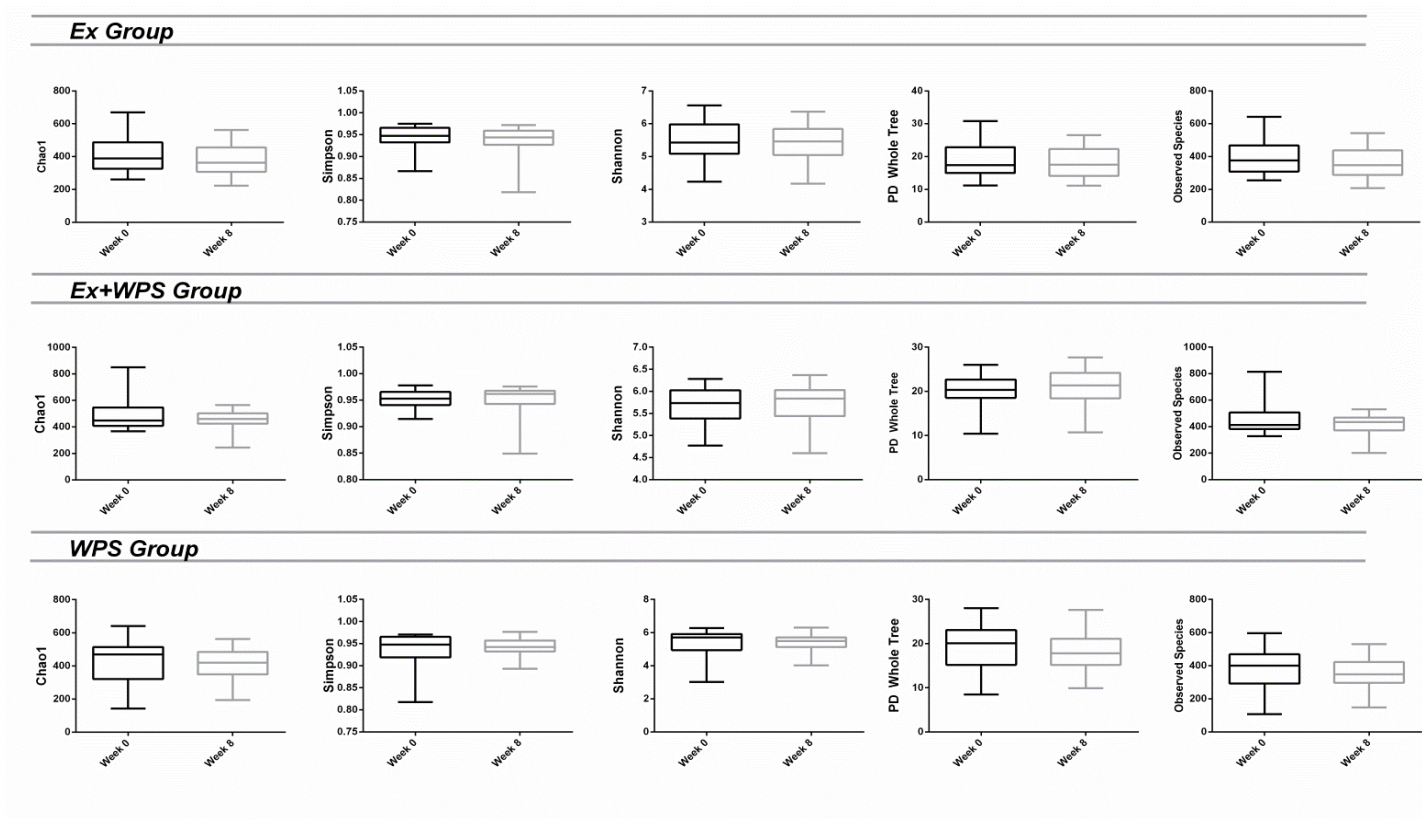


**Figure 2. An eight week exercise improves general fitness, while exercise, whey protein supplementation and a combination of both decreases resting blood pressure.** Statistically significant differences determined between baseline and post intervention anthropometric measurements determined by paired t-test where significance was taken as  $p \leq 0.05$ .

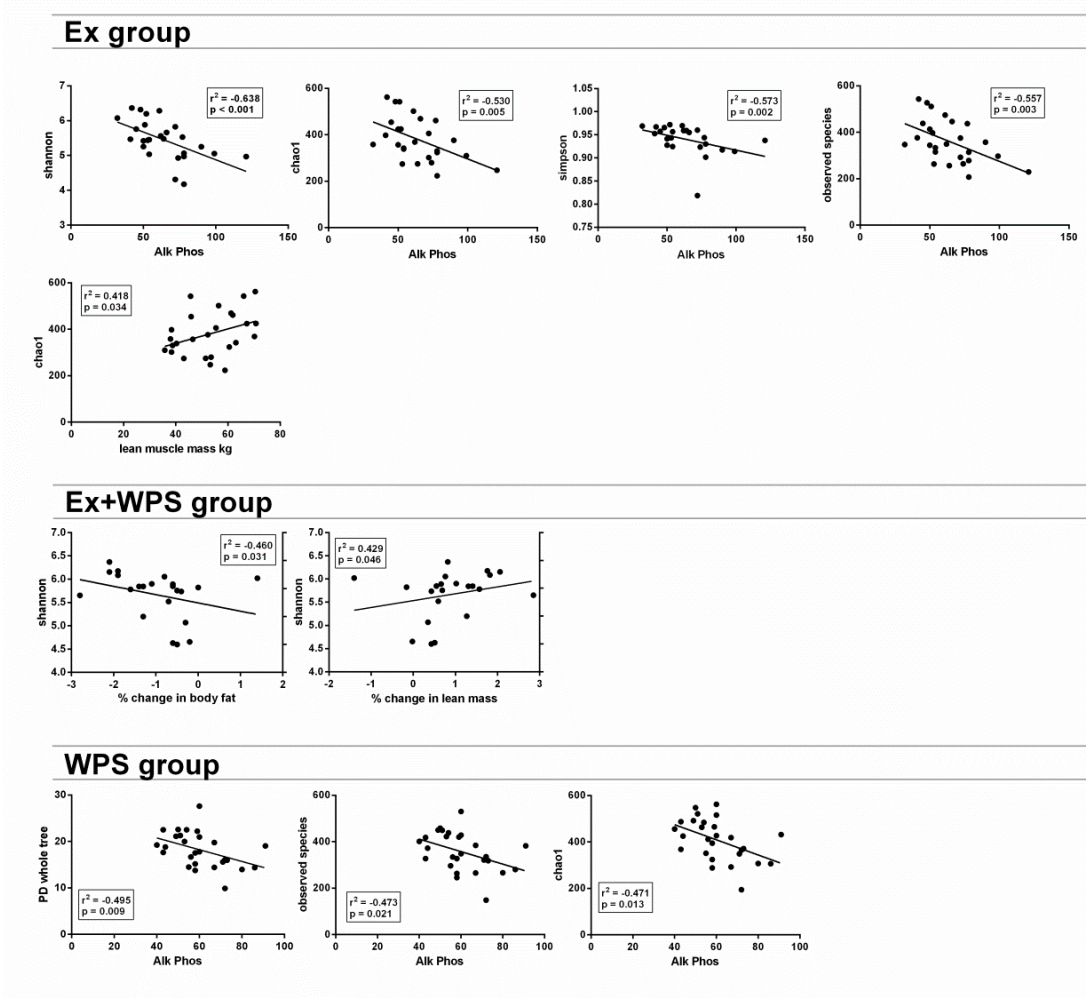




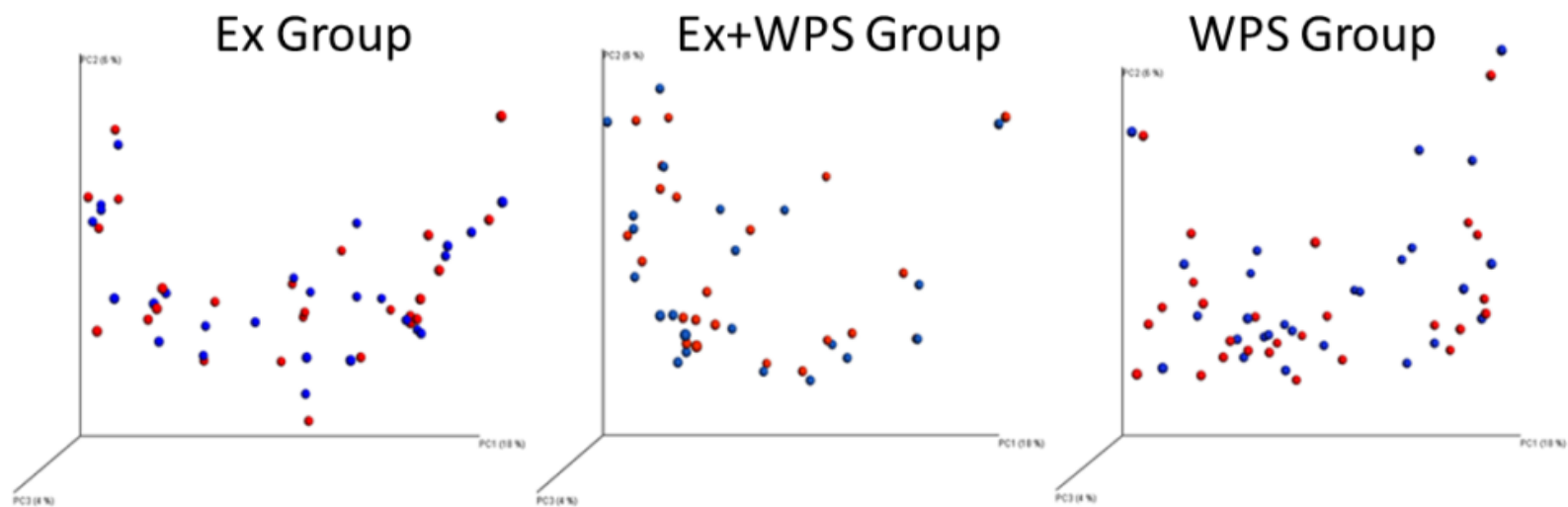
**Figure 4. Alpha diversity is unchanged by 8 weeks of exercise, whey protein supplementation or a combination of both.** Comparison of pre and post intervention assigned alpha diversity scores for each group within the study. Data is represented as mean  $\pm$  SEM.



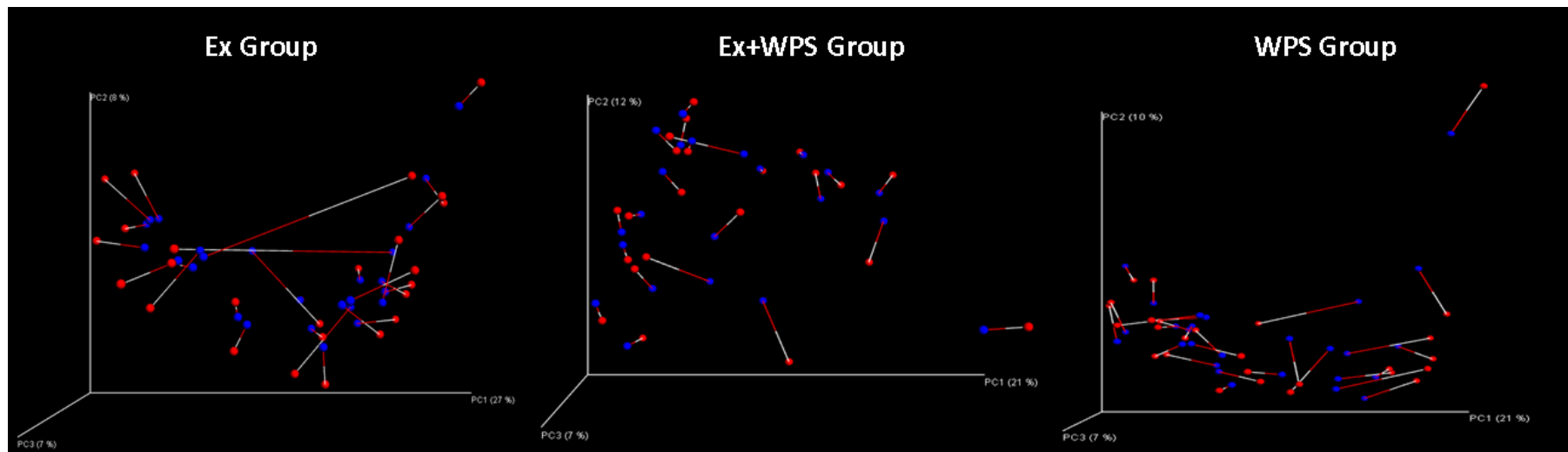
**Figure 5. Statistically significant Spearman correlations determined between post intervention anthropometric measurements, blood profile data and alpha diversity indices**



**Figure 6. Principal Coordinate analysis (PCoA) based upon unweighted Unifrac distances shows no separation between pre and post intervention sequence data.** PCoA analysis of each of the enrolled subjects' pre and post intervention 16S rRNA sequence data where • represents baseline (week 0) data and • represents post intervention (week 8) data.

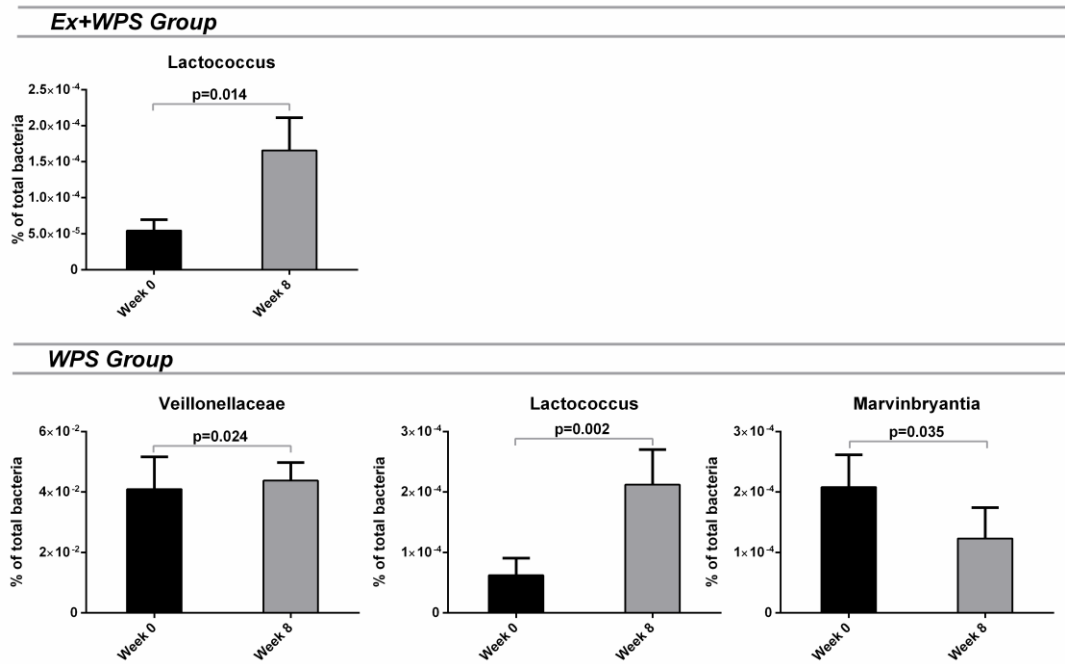


**Figure 7. Procrustes imaging of unweighted Unifrac distances matrices derived from pre and post intervention data from each subject within the study, reveal no uniform clustering pattern.** Individuals pre and post intervention data, where ● represents baseline (week 0) data and ● represents post intervention (week 8) data with flux in diversity illustrated by the connecting line.

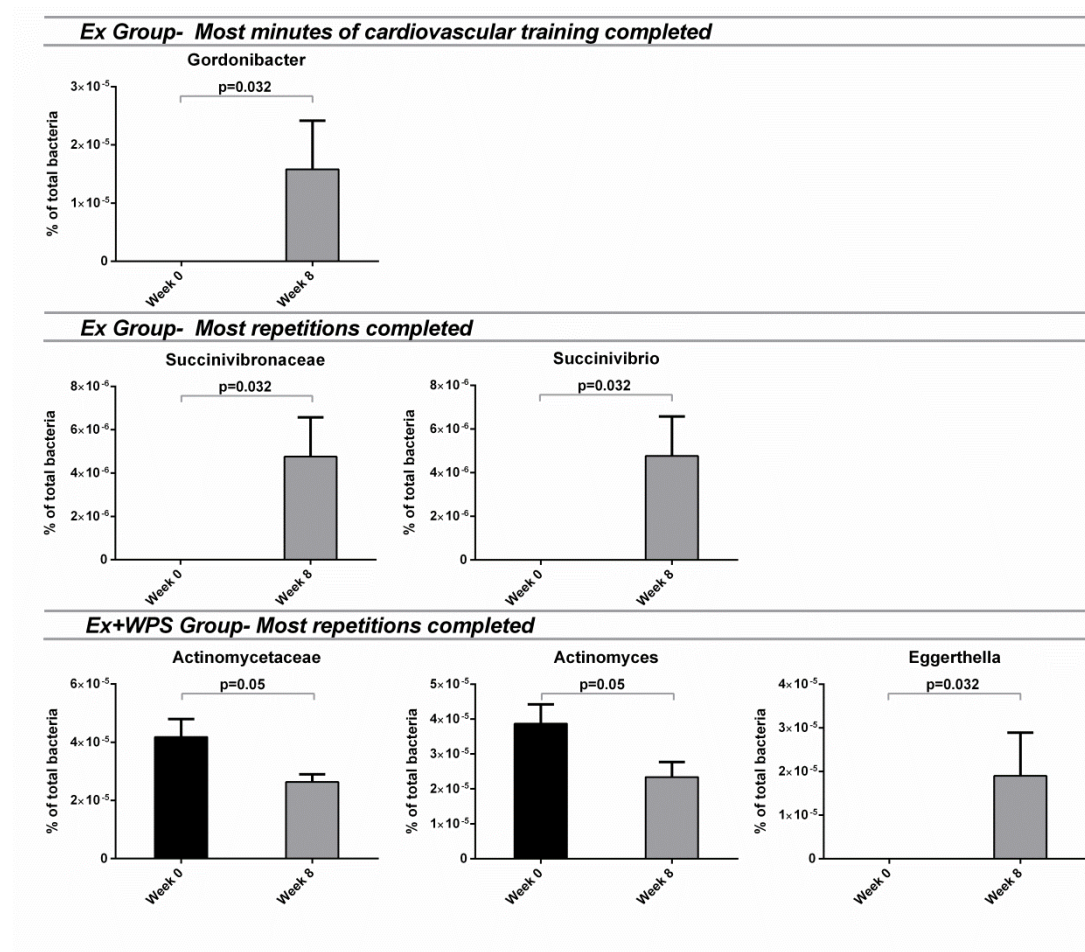


**Figure 8. Whey protein supplementation increases *Lactococcus* populations.**

Statistically significant differences in microbial taxonomies between individuals assigned to all three groups within the study’s pre and post intervention sequence data. Data is represented as a % of total bacteria  $\pm$  SEM where significance was taken at  $p \leq 0.05$ .



**Figure 9. The amount of exercise completed differentially alters enteric microbial taxonomies.** Statistically significant differences in microbial taxonomies determined between pre and post intervention period of the eight individuals assigned to the Ex and Ex+WPS groups which completed the most exercise both in terms resistance and cardiovascular training. Data is represented as a % of total bacteria  $\pm$  SEM where significance was taken at  $p \leq 0.05$ .





## General Discussion

The composition, diversity and functionality of the gut microbiota play a critical role in maintaining human health, with our microbial inhabitants and associated metagenome influencing several important physiological processes including energy absorption, regulation of fat storage and modulation of the host immune system (Sekirov, Russell et al. 2010). Conversely, perturbations to the composition or function of the gut microbiota have frequently been associated in the pathogenesis of a host of inflammatory GI diseases such as obesity and IBD (Marchesi, Adams et al. 2015). It is logical that selective manipulation of the gut microbiota can yield novel treatments for many such diseases (Walsh, Guinane et al. 2014). Diet is an environmental factor which has repeatedly been shown to be a major factor in driving the composition and metabolism of the gut microbiota (De Filippo, Cavalieri et al. 2010, Claesson, Jeffery et al. 2012, Scott, Gratz et al. 2013) and, therefore, diet represents one such tool by which gut microbial communities may be beneficially modulated. With respect to such dietary modulation, the vast majority of research to date has focused on the use of complex carbohydrates (prebiotics) such as fructooligosaccharides, whose fermentation by members of the gut microbiota primarily results in the formation of SCFAs (e.g. butyrate) which confer several health benefits to the host (Macfarlane, Steed et al. 2008, Flint, Scott et al. 2012, Dehghan, Gargari et al. 2014). However, the effect of other dietary components, such as protein, on the gut microbiota have received considerably less attention. Indeed dietary protein has been associated with the formation of putrefactive metabolites such as amines and N-nitroso compounds which may be detrimental to gut health (Windey, De Preter et al. 2012, Louis, Hold et al. 2014). Whey is the milk serum which remains after the casein curd has been removed during cheese

manufacture and accounts for ~20% of the total protein content of bovine milk. Whey is comprised of several component proteins such as  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, serum albumin, lactoperoxidase, and lactoferrin whose properties have been shown to beneficially influence several physiological processes such as energy balance and glucose metabolism (Pal, Ellis et al. 2010, Shertzer, Woods et al. 2011). While the consumption of whey protein(s) has/have been shown to have beneficial outcomes in disease states in which perturbations of the gut microbiota have been noted, such as obesity and T2D, their effect on the composition of gut microbial communities remains largely unknown.

Like diet, exercise is another environmental factor which influences a wide range of physiological processes and has become increasingly recognised as an effective alternative to pharmaceutical treatments for several disease states (Schwingshackl, Missbach et al. 2014). Given the capacity for exercise to influence intestinal conditions, it seems likely that exercise also influences the gut microbiota. However, as has been the case for whey protein, the impact of exercise on the gut microbiota is currently poorly understood. Notably, a recent study by Clarke *et al.* showed that professional rugby players harbour a significantly more diverse gut microbiota (indicator of gut health) than high or low BMI controls and it was also noted that a correlation existed between each of exercise and protein intake with this greater diversity (Clarke, Murphy et al. 2014). The aim of this thesis was, through *in vitro* and *in vivo* experimentation, to employ next generation sequencing technologies to investigate the effects of dietary whey protein (and its component proteins) and exercise on the composition and diversity of the gut microbiota.

As previously stated, the impact of dietary whey protein on the gut microbiota is poorly understood. Prior to the commencement of this study, it had been shown by Sprong *et al.* that dietary whey protein protected rats against induced colitis in that dietary whey was observed to increase mucin production and improve markers of inflammation (associated with GI disorders such as obesity and IBD). Furthermore, the same study reported that dietary whey protein increased fecal lactobacilli and bifidobacteria counts (Sprong, Schonewille et al. 2010). Unlike this previous study, which used qPCR to quantify fecal bacterial counts, our initial study (Chapter 2) employed high-throughput sequencing to more comprehensively assess the impact on the gut microbiota of C57BL/6J mice fed a high fat diet, with either WPI or casein as a protein source, for 21 weeks. Results from this study revealed that, when compared to mice consuming a high fat diet where casein was the protein source, the gut microbiota of WPI-fed animals contained significantly higher proportions of *Lactobacillus* and *Bifidobacterium*. With respect to obesity and other GI conditions characterised by inflammation, our results suggest that dietary WPI can confer health benefits to the host by enriching populations which exhibit anti-inflammatory properties (Imaoka, Shima et al. 2008, Liu, Fatheree et al. 2010). It was also notable that the body weights of mice in receipt of the high fat/whey protein diet were significantly lower than those of the high fat/casein diet and thus changes to microbial taxonomies may result from this significant reduction in body weight, which has been shown in other studies to alter the gut microbiota (Duncan, Lopley et al. 2008, Ravussin, Koren et al. 2012). Ultimately, while the question of cause versus effect remains, this study provides a valuable initial insight into the impact of dietary WPI consumption on the composition of the gut microbiota.

While results from our initial study delivered promising insights/findings relating to the impact of whey protein on weight gain and, potentially, the gut microbiota, it is not possible to determine the individual contributions of the large array of proteins and associated bioactive peptides contained within whey (Pihlanto-Leppälä 2000). Therefore the subsequent chapter (Chapter 3) represents a logical extension of Chapter in that it describes an investigation of the impact some of the component proteins of whey on body weight and the gut microbiota. More specifically, this study employed a two armed approach to investigate the effect of two component proteins of whey, namely BSA and Lf, on the composition and diversity of 1) the human gut microbiota by means of an *ex vivo* anaerobic fermentation system and 2) the gut microbiota of C57BL/6J mice in receipt of a high fat diet. In each case high-throughput DNA sequencing was employed to determine microbial composition. Our study, which was the first to use 16s rRNA gene sequencing to evaluate the effect of component proteins of whey on the murine enteric microbiota, revealed that replacement of casein as the protein source of a high fat murine diet with either Lf or BSA reversed several of the high fat associated taxonomic changes to the gut microbiota. Interestingly, with respect to Lf, these taxonomic changes were independent of a significant decrease in the animals' body weight. Furthermore, replacement of casein with Lf in a high fat murine diet was shown to significantly increase the proportions of the *Roseburia* genus, representatives of which, such as *Roseburia intestinalis*, have recently been proposed as new targets for prebiotic treatments owing to their butyrate producing capabilities (Duncan, Hold et al. 2002, Conlon and Bird 2014). To predict the effects that this altered microbiota might have with respect to gut microbiota functionality, we employed the PICRUST pipeline/application to predict the metabolic pathways that are encoded therein. This

approach revealed that the functional potential of the microbiome of both Lf and BSA fed animals was more akin to that of animals consuming a low fat diet than those consuming a high fat diet where casein was the protein source. This result suggests that as well as invoking taxonomic alterations, both Lf and BSA beneficially alter the functionality of the microbiota, which is regarded by many as being even more important with respect to its influence on health than microbiota composition (Turnbaugh, Hamady et al. 2009). The possibility that Lf and BSA can improve gut health, and reverse high fat diet associated changes to the composition and function of the gut microbiota, in the presence of a high fat diet is particularly relevant given that weight regain remains the most common long term outcome of weight loss programs (Bray and Wadden 2015). While these results suggest a use for bovine whey proteins (particularly Lf) in the beneficial modulation of the gut microbiota, further investigation into the mechanisms by which these dairy proteins bring about alterations to microbial taxonomies and the identification of the bacterial metabolites produced as a consequence of their fermentation is required.

As previously stated, exercise is an environmental factor which has been shown to influence several physiological processes. However its effects on the gut microbiota remain unclear (O'Sullivan, Cronin et al. 2015). Our study (Chapter 4) aimed to determine the effect of voluntary exercise on energy balance, metabolic activity and the composition of the gut microbiota through the use of a C57BL/6J murine model. To negate diet induced variability, such as high fat diet associated inflammation (Todoric, Löffler et al. 2006, Cani, Bibiloni et al. 2008), and to better assess the direct impact of exercise on bodily process and the gut microbiota, both control and exercising animals were fed the same low fat chow for the eight week study period.

Our results showed that, over the eight week intervention period, voluntary exercise significantly increased the animals' energy intake and metabolic activity while invoking subtle changes to the gut microbiota. Notably at the study's week four mid-point, Illumina MiSeq 16s rRNA gene sequencing of the murine fecal microbiota revealed that the gut microbiota of exercising animals harboured significantly higher proportions of the *Bacteroides* genus and showed significantly higher number of metabolic pathways for fatty acid biosynthesis (as determined by PICRUSt). At the trial's end point, hypothalamic gene expression analysis revealed that exercising animals showed a trend towards increased expression of genes involved in fatty acid biosynthesis and subsequent glucose uptake. Therefore, it may be that exercise invokes initial changes to the gut microbiota which in turn influence host gene expression profiles. Our findings support those from previous short term trials that have shown a similar subtle effect of exercise. (Allen, Miller et al. 2015). Furthermore, while the results of this study suggest subtle changes to the gut microbiota induced by exercise, as this was a short term study (eight weeks duration) perhaps a longer intervention period could result in more substantial exercise-driven changes to the gut microbiota. Indeed, a previous study in which a 12 week intervention period was employed showed, using PCoA analysis of 16s rRNA gene sequence data, a clear separation in the microbiotas of exercising and sedentary mice (Evans, LePard et al. 2014). Thus further studies over a variety of intervention periods are required to fully elucidate the exercise induced changes on the gut microbiota.

The gut microbiota of professional athletes was previously found to be significantly more diverse than that of control subjects and that this increased diversity was correlated with both exercise and protein consumption (Clarke, Murphy et al. 2014).

This observation, together with data from this thesis relating to both *in vivo* and *in vitro* experiments investigating the individual effects of whey, whey protein constituents and exercise on the gut microbiota and functionality, prompted a final study to examine the combined effects of altered diet and exercise on gut microbiota in a human trial. In this final study (Chapter 5), high-throughput DNA sequencing was employed to determine if an eight week exercise intervention and/or whey protein supplementation would influence the gut microbiota of previously physically inactive individuals whilst maintaining habitual dietary habits. Results from the study revealed that while completion of an eight week exercise program increased the general fitness of subjects, exercise did not invoke alterations to the composition or diversity of the gut microbiota. Similarly an eight week daily whey protein isolate supplementation alone or in combination with exercise did not alter the diversity of the subjects' microbiota. This result suggests that the large inter-individual dietary variations existing between the subjects within the trial reduces the impact of WPI supplementation of the gut microbiota and suggests that further studies should also consider other components of diet within the study design. Akin to our own observations from Chapter 4, the short intervention period employed presently appears to also seem to be a limiting factor in exercise's ability to considerably influence the gut microbiota.

Ultimately, the importance of the composition, diversity and functionality of the gut microbiota with respect to human health is now clearer than ever. Developments in culture independent technologies over the past decade have allowed for a rapid expansion in our understanding of these microbial communities and the environmental factors which influence their composition. Through the use of high-throughput next generation sequencing technologies, this thesis provides valuable

and novel insights into the impact of both dairy whey proteins and exercise on the enteric microbial communities.



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